THE INVESTIGATION OF AT7519 (CDK INHIBITOR) AND AT13387 (HSP90 INHIBITOR) AS NOVEL THERAPIES FOR THE TREATMENT OF PANCREATIC DUCTAL ADENOCARCINOMA

Thesis submitted in accordance with the requirements of the university of Liverpool for the degree of Doctor in Medicine by Amy Jayne Thomas

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DEDICATION

FOR JACK

The only man in my life who had the good grace to cause no trouble until I'd finally finished drafting this thesis apart from the occasional kick of encouragement.

DECLARATION

This thesis is the result of my own work except where technical assistance and collaboration with colleagues is acknowledged.

Collaboration was undertaken on investigation of these agents with Elisabeth Shaw (Post-doctorate researcher) and Owain Jones (MD student). The following experiments where primarily performed by Elisabeth Shaw and included in this thesis: IC50 work for Fampac cell line, cell line photography for IC50 work, cell cycle analysis experiments, apoptosis experiments, western blots for AT13387. Owain Jones performed the westerns using NPM, pNPM antibodies. All other experimental data shown here was primarily undertaken by myself.

The material contained in this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification.

The research work presented in this thesis was carried out in the Department of Molecular and Clinical Cancer Medicine and the Roy Castle Cancer Research Center, University of Liverpool, Liverpool, United Kingdom from August 2009 to August 2012.

Amy Jayne Thomas

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ABSTRACT

THE INVESTIGATION OF AT7519 (CDK INHIBITOR) AND AT13387 (HSP90 INHIBITOR) AS NOVEL THERAPIES FOR THE TREATMENT OF PANCREATIC DUCTAL ADENOCARCINOMA

Author: Amy Jayne Thomas MBChB, MRCS (Eng)

BACKGROUND: Pancreatic ductal adenocarcinoma (PDAC) is usually a systemic disease and so requires chemotherapy even when surgical resection is possible. Current agents such as gemcitabine are only effective in a minority of patients and chemoresistance is an important issue faced in pancreatic cancer. There is an urgent need for more effective therapies; Novel agents may offer an avenue for this. The CDK family of protein kinases are pivotal in cell cycle regulation that is often deranged in cancer. HSP90 is a molecular chaperone affecting multiple key cellular signaling pathways of importance in pancreatic cancer.

OBJECTIVES: To determine the efficacy of AT7519, a novel CDK inhibitor, and AT13387, a novel HSP90 inhibitor in pancreatic cancer models in vivo and in vitro both as single agents and in combination with gemcitabine.

METHODS: Cell proliferation was measured using the EZ4U assay. Cell cycle analysis was performed with flow cytometry. Apoptosis analysis was using the Caspase-Glo 3/7 luminescent assay. Western blotting assessed expression of client proteins and phosphorylation. A murine xenograft model was employed assessing tumour volumes with external calipers. Experiments were performed using AT7519 and AT13387 as single agents and in combination with gemcitabine.

RESULTS: AT7519 inhibited proliferation in cell lines including a gemcitabine resistant line (SUIT-2 GR) with IC50 values ranging 5-2000nM. Cell cycle analysis showed actions in line with CDK inhibition. At 24 hours induction of caspase 3/7 was significantly increased in AT7519 treated cells compared with those in control media ($p=0.01$). Phosphorylation of client proteins were inhibited. Isobolar analysis of AT7519 combined with gemcitabine suggested an additive effect. AT7519 was tolerated as a single agent and in combination with gemcitabine in a xenograft model and resulted in slowed tumour growth compared to control ($p = 0.0446$).

AT13387 inhibited proliferation of all cell-lines with IC50 values of 29nM-325nM including a cell line with acquired gemcitabine resistance. AT13387 treated cells accumulated in G0/G1 and G2/M phases of the cell cycle ($p<0.05$). Late apoptosis was seen after 40 hours post treatment with At13387. AT13387 treatment resulted in down-regulation of HSP90 client proteins and up-regulation of the HSP70 co-chaperone. AT13387 was tolerated and moderately efficicacous as a singe agent in a murine xenograft model but was poorly tolerated in combination with gemcitabine and conferred no benefit over single agents.

CONCLUSIONS: AT7519 is a promising agent for combination therapy in pancreatic cancer, as acquired resistance to gemcitabine does not give AT7519 resistance.

AT13387 showed promising effects in vitro but was poorly tolerated in an in vivo combination with standard therapy therefore may offer a therapeutic avenue for non-responders to conventional therapies.

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ABBREVIATIONS

ATP	Adenosine triphosphate
CDK	Cyclin dependant kinase
DM	Diabetes Mellitus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
GI	Gastrointestinal
GIST	Gastroinestinal stromal tumour
GTP	Guanosine triphosphate
hCNT	Humaan concentrative nucleoside transporter
hENT	human equilibrative nucleoside transporter
HER2	Human epidermal growth factor
HSP70	Heat shock protein 70
HSP90	Heat shock protein 90
IC50	Half maximal inhibitory concentration
MAP	Mitogen-activated protein
mRNA	messenger RNA
NMR	Nuclear magnetic resonance
OS	Overall survival
PanIN	Pancreatic intraepithelial neoplasia
PBS	phosphate buffered saline
PDAC	Pancreatic ductal adenocarcinoma
PFS	progression free survival
Rb	retinoblastoma protein
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline

Tween	Polyoxyethylenesorbitan
UICC	Union for international cancer control
VEGF	Vascular endothelial growth factor
5-FU	Fluorouracil

UNITS

M	molar
mM	milimolar
µM	micromolar
nM	nanomolar

CHAPTER 1: INTRODUCTION

1.1 EPIDEMIOLOGY

Pancreatic cancer continues to a pose significant health problem being the 5th most common cause of cancer death in the UK. Despite current research and advances in understanding pancreatic cancer biology, imaging detection methods and improved surgical outcomes, the prognosis remains poor with a five-year survival of between 10-25% in resected disease(Richter, Niedergethmann et al. 2003; Cress, Yin et al. 2006). The mortality roughly equates to the incidence; in 2012 in the UK there were 8,888 new cases of pancreatic cancer and 8,622 deaths(UK 2014). Despite advances in many other cancers resulting in improved survival the incidence and mortality rates for pancreatic cancer have not seen any significant improvement over the last 30 years (Malvezzi, Bertuccio *et al.* 2012).

1.1.1 Age

Advancing age is associated with an increased incidence of pancreatic cancer. The peak incidence occurs at age 65-75(Lankisch, Assmus *et al.* 2002), less than 0.3% of cancers occur in those under 40 years(Luttges, Stigge *et al.* 2004). Comparing the incidence in different age groups shows in those over the age of 80 incidence is 100 per 100 000 population compared to 2 per 100 000 for those between 40 and 44.

1.1.2 Gender

Pancreatic cancer has an increased incidence in the male population compared with females overall, though there is some geographical variation. For men the age-standardised worldwide rate is 4.6 per 100 000 and 3.3 per 100 000 for females (ratio 1.4:1)(Sahmoun, D'Agostino *et al.* 2003).

1.1.3 Geography

Geographically the highest incidence for men is observed in Eastern Europe and North America, the lowest in southern/northern Africa and Southeast Asia. For women the highest incidence is seen in Northern Europe and North America, the lowest is the same as males in Africa and Southeast Asia (7).

1.2 AEITOLOGY

Risk factors for pancreatic cancer have been identified that include both genetic and environmental factors. Approximately 10% of pancreatic cancers are predominately due to a genetic predisposition associated with a range of syndromes and respective gene mutations(Giardiello, Brensinger et al. 2000; Lal, Liu et al. 2000; McWilliams, Rabe et al. 2005).

1.2.1 Familial Pancreatic Cancer

A number of large epidemiological studies have established an increased risk of cancer with a positive family history(Fernandez, La Vecchia et al. 1994; Klein, Brune et al. 2004; McWilliams, Rabe et al. 2005). Most of this elevated risk is probably due to multigenetic factors, but there is evidence for a rare autosomal dominant condition (Familial Pancreatic Cancer). Early studies demonstrated families with increased incidence but the onset of cancer was not early and histology and survival similar to sporadic cases (Ehrenthal, Haeger et al. 1987; Ghadirian, Boyle et al. 1991). From case-control and cohort studies it is clear that having a single-close relative with pancreatic cancer doubles an individuals lifetime risk of developing PDAC(Amundadottir, Thorvaldsson et al. 2004; Hassan, Bondy et al. 2007). Epidemiological studies have also shown that patients with a family history of pancreatic cancer also have an increased risk of extra pancreatic malignancies, but this is not necessarily true in the autosomal dominant syndrome. Wang *et al* demonstrated that relatives of patients with cases of pancreatic cancer have an increased risk of dying from breast cancer (RR1.66, 95% CI 1.15-2.34), ovarian cancer (RR 2.05, 95% CI 1.10-3.49) and bile duct adenocarcinoma (RR 2.89, 95% CI 1.04-6.39)(Wang, Brune *et al.* 2009).

1.2.2 Hereditary Cancer Syndromes

In most individuals with a predisposition for cancer the gene involved remains unknown, however a number of known genetic syndromes show associations with increased risk of pancreatic cancer summarised in table 1.1. PDAC is seen in some breast cancer families with BRCA1 and BRCA 2 mutations(Couch, Johnson *et al.* 2007) and BRCA2 may account for up to 5% of all pancreatic cancers(Goggins, Schutte *et al.* 1996). Patients with familial atypical multiple mole melanoma (FAMM) due to p16 gene mutations have a 9 to 47-fold increased risk of developing pancreatic cancer and pancreatic cancer is the second most common cancer in this syndrome(Lynch and Fusaro 1991; Vasen, Gruis et al. 2000). Peutz-Jeghers syndrome is an autosomal dominant syndrome characterised by harmartomatous polyps of the GI tract and melanocytic macules of the oral mucosa and lips. Patients with Peutz-Jeghers syndrome have a high risk of developing pancreatic cancer as well as other malignancies of the gastrointestinal tract and breast cancer(Boardman, Thibodeau et al. 1998; Hearle, Schumacher et al. 2006). Hereditary pancreatitis is a rare autosomal dominant condition characterised by recurrent and severe attacks of acute pancreatitis at a young age. Mutations in the PRSS1 gene have been shown to be causative in hereditary pancreatitis and patients have a lifetime risk of developing pancreatic cancer of up to 40%(Whitcomb, Gorry et al. 1996; Lowenfels, Maisonneuve et al. 1997).

Syndrome	Gene defect	Increased risk of PDAC
Hereditary breast and ovarian cancer	BRCA2, FANC-C, FANC-G, PALB2	3.5-10 fold
FAMM	P16	9 to 47 fold
Hereditary pancreatitis	PRSS1, SPINK1	50 to 80 fold
Peutz-Jeghers	STK11	132-fold
Li-Fraumeni	P53	unknown
Cystic fibrosis	7q31	2-61-fold

Table 1.1 Table showing the hereditary cancer syndromes affecting the pancreas with known gene defects and the noted increased risk of pancreatic cancer.

1.2.3 Chronic Pancreatitis

Chronic pancreatitis is a well-recognised risk factors for the development of pancreatic cancer. The cumulative 25-year risk of pancreatic cancer in individuals with chronic pancreatitis has been quoted at around 4%(Lowenfels, Maisonneuve *et al.* 1993). Lowenfels *et al* looked a large cohort of patients with chronic pancreatitis and showed a six times greater risk of development of pancreatic cancer in affected individuals(Lowenfels, Maisonneuve *et al.* 1993). In another large case-control study a history of chronic pancreatitis or acute pancreatitis for seven years or more confirmed an increased risk of pancreatic cancer (RR 2.03, 95% CI 1.53-2.72)(Bansal and Sonnenberg 1995).

1.2.4 Diabetes Mellitus

The association between pancreatic cancer and diabetes mellitus has been investigated in depth as both a causative factor and a feature of early disease. Large prospective cohort study in US investigated the independent association with post-load plasma glucose concentration and risk of pancreatic cancer mortality among individuals without self reported diabetes. This study concluded that the factors associated with abnormal glucose metabolism may play an important role in the aetiology of PDAC (Gapstur, Gann *et al.* 2000). A early meta analysis found a relative risk of 2.0 for pancreatic cancer in patients with at least a 5 year history of diabetes mellitus compared with non-diabetics(Everhart and Wright 1995). A recent meta analysis concluded that DM is both an early manifestation of pancreatic cancer and an etiologic factor(Ben, Xu *et al.* 2011).

1.2.5 Smoking

The causal link between smoking and pancreatic cancer has been demonstrated in a number of studies dating back almost as far as 30 years(Gordis and Gold 1984; Hirayama 1989). There is a clear dose response noted (Falk, Pickle *et al.* 1988) and risk can be reduced after a period of abstinence(Howe, Ghadirian *et al.* 1992). In some case-control studies the risk of PDAC rises with excessive lifetime cigarette consumption. Being a smoker and having a first degree relative with PDAC further increased the overall relative risk (RR6.02, 95% CI 1.98-18.29)(Ghadirian, Lynch *et al.* 2003).

Smoking and chronic pancreatitis have also been shown to be significant risk factors for the development of pancreatic cancer(Lowenfels, Maisonneuve et al. 1993; Nilsen and Vatten 2000).

1.2.6 Alcohol intake

The association between acute and chronic pancreatitis and heavy alcohol consumption has been well recognised but its link definitively to pancreatic cancer has been somewhat elusive. The study evidence was reviewed by a panel of experts in 2009 convened by the Agency for research on cancer (Secretan, Straif *et al.* 2009). Previous studies including cohort studies and meta-analyses of prospective studies reported significant association between pancreatic cancer risk and alcohol consumption(Heuch, Kvale et al. 1983; Heinen, Verhage et al. 2009; Michaud, Vrieling et al. 2010; Tramacere, Scotti et al. 2010) but others demonstrated no association(Isaksson, Jonsson et al. 2002; Rohrmann, Linseisen et al. 2009). These previous studies lacked convincing evidence owing to a number of factors including design of studies, potential confounding factors namely smoking and the associations limited in a number of studies by sample size, potential recall bias and selection bias. In 2011 the Cancer prevention study II published its findings into the relationship between alcohol consumption and pancreatic cancer. This was a large prospective cohort study with data from approximately 1.2 million subjects in the USA. This study confirmed that consumption of 3 or more drinks per day was associated with pancreatic cancer mortality in both non smokers and smokers specifically in spirit consumption but not wine or beer(Gapstur, Jacobs *et al.* 2011).

This study is the clearest to date to suggest heavy alcohol intake is associated independently with pancreatic cancer mortality.

1.2.7 Nutrition

Some studies have reported dietary factors as possible risk factors for the development of PDAC. Increased intake of meat, dairy products, eggs, milk, fried food and salt have been suggested though thus far an independent causative role has not been proven not least owing to the difficulties of isolating nutritional factors as independent causative agents (Ghadirian, Lynch *et al.* 2003).

1.2.8 Obesity

There have been large cohort studies investigating the relationship between pancreatic cancer and obesity. Results have shown that a Body mass index of 30 or greater had an increased risk of cancer compared with patients with a BMI of 23 (RR 1.72, CI 95% 1.19-2.48) (Michaud, Giovannucci *et al.* 2001). In a UK meta analysis by cancer research there was a weak association between obesity and pancreatic cancer (RR1.19, 95% CI 1.1-1.29)(Berrington de Gonzalez, Sweetland *et al.* 2003).

1.2.9 Previous surgery

Studies into the link between previous surgery and pancreatic cancer has provided conflicting evidence. One study showed an increased risk of PDAC after gastrectomy for peptic ulcer disease (RR1.8, 95% CI 1.3-2.6) but no association was evident in another Italian study(Tascilar, van Rees *et al.* 2002)

1.3 HISTOPATHOLOGY

Over recent times there has been research that has provided a greater understanding of the molecular basis of pancreatic cancer and histopathological processes underpinning its development.

1.3.1 PanIN Lesions

The development of pancreatic cancer has been well described as a stepwise process involving precursor lesions named pancreatic intraepithelial neoplasia (PanIN) which has resulted in a model of progression of these lesions to pancreatic ductal adenocarcinoma shown in figure 1.1. A classification based on morphologic features as been developed with three grades of PanIN lesions: PanIN-1, PanIN-2 and PanIN-3, it as been noted that PanIN-3 lesions occur in association with PDAC in around half of cases histologically examined, supporting the concept that high-grade PanIN lesions are precursor lesions for PDAC. PanIN lesions are found in the smaller pancreatic ducts (<5mm) where the usual cuboidal flat epithelial lining is

replaced by columnar mucinous cells. PanIN-1 lesions are classified as PanIN-1A or PanIN-1B. In PanIN-1A the mucinous epithelium is flat and in PanIN-1B papillary mucinous epithelium is present, both have minimal cytonuclear atypical appearances. In PanIN-2 lesions there is a greater degree of cytonuclear atypia and appearances consistent with low-grade dysplasia and in PanIN-3 lesions complex papillary architecture, mitotic figures, nuclear pleomorphism and at times necrosis resulting in appearances consistent with high-grade dysplasia or carcinoma –in-situ. In PanIN-3 lesions there is no invasive growth and the lesion is still combined within the basement membrane(Hruban, Adsay *et al.* 2001).

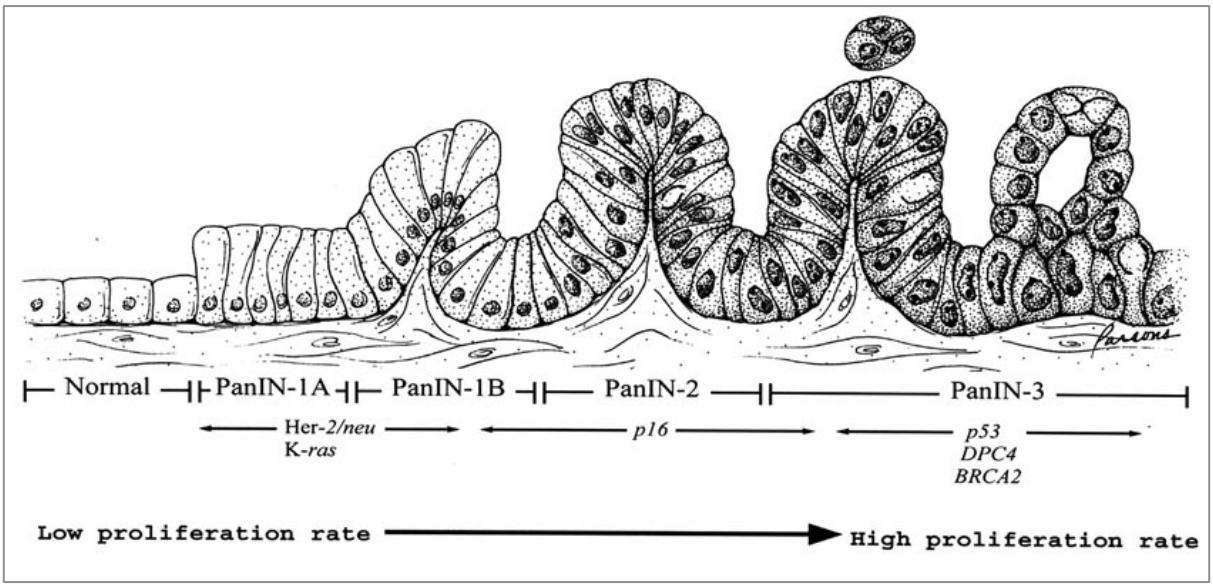


Figure 1.1 Figure showing the progression model of pancreatic cancer (Klein, Hruban et al. 2002)

Evidence for PanIN lesions being precursor lesions for PDAC comes from histological analysis and common genetic mutations in these lesions and PDAC. In autopsy

studies an increased prevalence of PanINs was documented with increasing age corresponding with increased rates of PDAC(Cubilla and Fitzgerald 1976). Further studies examined resection specimens of PDAC revealing presence of PanINs in the area surrounding a tumour. PanIN-1 were found in 75%, PanIN-2 in 65% and PanIN-3 in 50%(Andea, Sarkar *et al.* 2003) and Brat *et al* presented 3 cases of patient with documented PanIN lesions that went on to develop PDAC(Brat, Lillemoe *et al.* 1998). The genetic and epigenetic mutations commonly found in PDAC are discussed in later chapters but it is noted that common mutations occur in PanINs. Kras2 mutations are found in up to 85% of PanIN-3 lesions(Lohr, Kloppel *et al.* 2005) and mutation of the important tumour suppressor gene p16 has also been documented in PanIN lesions(Wilentz, Geradts *et al.* 1998). Positive immunostaining has been shown as a surrogate marker of mutation and inactivation of the p53 tumour suppressor gene and has been demonstrated in PanIN lesions(Baas, Mulder et al. 1994; Maitra, Adsay et al. 2003) and SMAD4 tumour suppressor gene mutations and BRCA2 mutations have also been identified in PanIN-3 lesions(Wilentz, Iacobuzio-Donahue *et al.* 2000).

The transition of high-grade PanIN to PDAC is still an area of investigation and the genetic alterations that may play a role in this transition are still not clearly understood or mostly unknown(Murphy, Hart et al. 2013). Investigation of genetic data has suggested there is nearly a 12 year window from the earliest precursor lesion changes to PDAC in a mathematical model(Iacobuzio-Donahue 2012) so clearly this raises the possibility of changing genetics of lesions as they develop and

also suggests a significant window of opportunity to modify these changes as they are further understood in the future(Brosens, Hackeng et al. 2015).

1.3.2 PDAC

Pancreatic ductal adenocarcinoma is the most common malignancy found in the pancreas. Solid tumours with poorly demarcated edges being characteristic. The majority of PDAC tumours are well to moderately differentiated formed by tubular or duct-like structures resulting from mucinous columnar cells. The tubular structures are lined by a single layer of epithelia cells sometimes showing papillary projections. Macroscopically this demonstrates poorly demarcated nodular growth with areas of necrosis or haemorrhage and a marked desmoplastic reaction in the surrounding tissues. In moderately and poorly differentiated tumours there is increasing cellular irregularity poorly formed glands and decreased mucous production. There are further malignant tumour types of the pancreas which are closely related to PDAC and considered variants of PDAC though these are less common, as well as other malignant solid and cystic neoplasms all with varying prognoses. Table 1.2 summaries the various histological types seen.

Histological tumour type	Frequency	Prognosis
Solid Tumours		
Ductal adenocarcinoma and variants	90%	Poor
Acinar cell carcinoma	1%	Poor
Pancreatoblastoma	<1%	Poor
Endocrine tumours	2%	Intermediate*
Non-epithelial tumours	Rare	Intermediate*
Cystic tumours		
Intraductal papillary mucinous neoplasms	2%	favourable
Mucinous cystic neoplasms	1%	favourable
Serous cystic neoplasms	1%	favourable
Solid psuedopapillary neoplasm	<1%	favourable
Other cystic tumours	1%	Intermediate*
Nonepithetial lesions and tumours	Rare	Intermediate*

*dependent on subtype

Table 1.2 The general features of pancreatic tumours. Adapted from the pancreas an integrated textbook of basic science, medicine and surgery second edition Beger et al Blackwell publishing 2008(Beger 2008).

At the time of diagnosis there is often local dissemination of disease with perineural invasion and spread to the peripancreatic fatty tissue almost universal and commonly invasion of lymphatic vessels, veins and nerves.

Histological analysis of tumours can be undertaken with cytokines 7, 8, 18 and 19, CEA and MUC1, these remain important though not specific to PDAC and conversely negative staining for neuroendocrine markers and pancreatic enzymes are helpful diagnostically(Batge, Bosslet et al. 1986; Takeda, Nakao et al. 1991). Genetic

mutations in Kras, p16, p53 and SMAD4 can often be demonstrated (discussed in section 1.4) as well as overexpression of EGFR and HER2 and less commonly VEGF(Apple, Hecht et al. 1999; Sipos, Weber et al. 2002)

1.3.3 Histopathological grading and staging

Kloppel *et al* developed a system of grading pancreatic tumours that encompassed the variable histological characteristic of tumours between tumours and within individual tumours initially in 1985(Kloppel, Lingenthal *et al.* 1985). Since then other grading systems have been employed. The Japan Pancreas society described a more complex system assigning grades for a variety of tissue structures (mizumoto 1996).

The UICC recommendation is that the least favourable grade recorded in an individual tumour should be that assigned(Sobin and Fleming 1997) and the International pancreatic cancer study group has recommended that the predominant grade and any other observed should be recorded. From the various recommendations it remains true that all areas of the tumour should be examined as accurate grading remains important and can give prognostic information (table 1.3).

Histopathological grading	
GX	Grade of differentiation cannot be assessed
G1	Well differentiated
G2	Moderately differentiated
G3	Poorly differentiated
G4	Undifferentiated

Table 1.3 The histological grading of PDAC, adapted from Guzman et al (Guzman G 2007)

The American Joint Committee on Cancer (AJCC) TNM classification is the most widely used system to stage PDAC and describes the anatomical extent of the cancer in relation to the local tumour, lymph node metastasis and any distant metastasis(Wolfgang, Herman et al. 2013) (table 1.4).

T - Primary Tumour	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ*
T1	Tumour limited to pancreas, 2cm or less in greatest dimension
T2	Tumour limited to pancreas, more than 2cm in greatest dimension
T3	Tumour extends beyond pancreas, but without involvement of coeliac axis or superior mesenteric artery
T4	Tumour involves coeliac axis or superior mesenteric artery
N - Regional Lymph Nodes	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
pN0	Lymph nodes negative but number ordinarily examined are not met (10 or more)
N1	Regional lymph node metastasis
M – Distant Metastasis	
M0	No distant metastasis
M1	Distant metastasis
pM1	Distant metastasis microscopically confirmed

Note: *Tis also includes the 'PanIN-III' classification.

Note: The MX category is considered to be inappropriate as clinical assessment of metastasis can be based on physical examination alone. (The use of MX may result in exclusion from staging).

Table 1.4 Table showing thr TNM and pTNM classification of PDAC which provides a general anatomical staging of disease, adapted from National Comprehensive Cancer Network

http://www.nccn.org/professionals/physician_gls/pdf/pancreatic.pdf

The TNM staging describes the cancer without considering treatment, this staging can be supplemented with the R classification which describes tumour status after treatment therefore reflecting the effects of therapy and giving a stronger predictor of prognosis (table 1.5).

In PDAC lymph node involvements has been shown as a negative prognostic indicator in multivariate analysis(Ferrone, Finkelstein et al. 2006; Shimada, Sakamoto et al. 2006; Winter, Cameron et al. 2006; Schnelldorfer, Ware et al. 2008). Resection margin involvement is also considered of prognostic significance in PDAC. In the literature R1 rate (positive resection margins) vary significantly and the rates of margin involvement and local recurrence often variable (Benassai, Mastrorilli et al. 2000; Jarufe, Coldham et al. 2004; Han, Jang et al. 2006; Moon, An et al. 2006; Sierzega, Popiela et al. 2006). Guidance from the royal college of pathologists has stated for PDAC when there is carcinoma present less than 1mm from the resection margin the margin should be considered positive i.e. R1 and for the anterior surface of the pancreas 0mm would be appropriate as this is an anatomical surface rather than a surgical resection margin(Campbell, Smith et al. 2009; Van den Broeck, Sergeant et al. 2009).

Residual Tumour (R) Classification*	
RX	Presence of residual tumour cannot be assessed
R0	No residual tumour
R1	Microscopic residual tumour
R2	Macroscopic residual tumour
RX	Presence of residual tumour cannot be assessed

Note: *some consider the R classification to apply only to the primary tumour and its local or regional extent. Others have applied it more broadly to include distant metastasis. The specific usage should be indicated when the R is used

Table 1.5 The residual tumour (R) classification of pancreatic tumours which classifies cancer status after treatment, adapted from Wittekind et al (Wittekind CH 2003).

An overall staging of disease based on the TNM status provides a standardized classification system to determine appropriate treatment options and provide prognostic information from the time of diagnosis (table 1.6). Resectable disease is classed as stage II, borderline stage III and I and unresectable or advanced is stage IV. This classification system allows clinician to determine the best treatment options moving forward and also prognostic information.

Stage Grouping			
Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T2	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T1, T2, T3	N1	M0
Stage III	T4	Any N	M0
Stage IV	Any T	Any N	M1

Table 1.6 table showing the overall stage groupings of PDAC based on the TNM classification, adapted from National Comprehensive Cancer Network http://www.nccn.org/professionals/physician_gls/pdf/pancreatic.pdf

1.4 CANCER MOLECULAR BIOLOGY

1.4.1 Alterations in oncogenic pathways in pancreatic cancer

Known molecular pathways in pancreatic cancer that promote tumourigenesis are related to a number of oncogenes and tumour suppressor genes.

1.4.2 Kras mutations

The most common mutation found in PDAC is that of the oncogene *KRAS*, reported mutated in up to 90% of pancreatic cancers(Almoguera, Shibata et al. 1988; Hruban,

van Mansfeld et al. 1993). Mutations of *KRAS* affect codon 12 primarily but aberrations have also been found in codons 13 and 61 (19). The mutant *KRAS* expresses a protein (Kras) that does not self inactivate and so is suspended in the GTP-bound state which stimulates a number of downstream signalling cascades in the development and progression of PDAC(Malumbres and Barbacid 2003).

1.4.3 Growth factor receptors

Inappropriate expression of a variety of growth factor receptors are also deemed important in PDAC. Epidermal growth factor receptor is a member of the ErbB family of receptor tyrosine kinases that has been demonstrated to be over expressed in PDAC(Korc, Chandrasekar *et al.* 1992) along with the principal natural ligands for EGFR, epidermal growth factor and transforming growth factor-α. EGFR causes receptor homo or heterodimerisation by binding ligands to the extracellular domain. This results in phosphorylation of tyrosine residues on the intracellular domain providing docking sites for mediators activating downstream signalling pathways: including PI3K/Akt signalling pathway (mediating cell cycle progression and survival); Ras-Raf-MEK pathway (transmitting growth signals) and other pathways that facilitate a number of functions advantageous for cancer cell survival(Marshall 2006). A variety of other growth factor receptors and their ligands are expressed in PanIN lesions and PDAC including insulin-like growth factor and vascular endothelial growth factor (VEGF) receptor promoting endothelial cell proliferation and survival, thereby enhancing tumour angiogenesis. VEGF expression

(mainly VEGF-A) is noted in up to 90% of pancreatic cancers and confers poor survival when at moderate/high levels(Seo, Baba *et al.* 2000).

1.4.4 p16

Aberrant p16 expression and namely alterations in the $p16^{INK4A}$/retinoblastoma protein pathway affecting tumour suppressor genes has also been identified in PDAC. In PDAC the pathway is abrogated through functional inactivation of the INK4A gene, this results in a lack of the normal suppression of phosphorylation of Rb that blocks entry into S phase and mitogenic signals in the cell cycle(Caldas, Hahn et al. 1994; Rozenblum, Schutte et al. 1997).

1.4.5 p53

The *TP53* tumour suppressor gene is the most commonly inactivated gene in cancers in general and more than 50% of pancreatic cancers (Waddell, Pajic et al. 2015).. p53 is usually activated in response to a range of cellular stresses and regulates transcription leading to cell cycle arrest or apoptosis. Mutations of p53 are thought to occur in the latter PanIN lesions and aberrant p53 to contribute to the extensive genetic instability that characterises PDAC(Hingorani, Wang *et al.* 2005).

1.4.6 Smad4

Mutations of *SMAD4*, originally identified as a tumour suppressor gene in pancreatic cancer are more common in this setting than in any other cancer. Smad4 lies downstream to the polypeptide growth factor TGF-β which affects cell growth, differentiation, angiogenesis and invasion. Loss of Smad4 has been shown to lead to amelioration of TGF-β tumour suppressive functions and maintenance of TGF-β mediated tumour-promoting functions(Levy and Hill 2005).

1.4.7 Notch pathway

The Notch pathway, which is important developmentally in the pancreas, is also activated in the early stages of PDAC and is important in invasion(Leach 2005; Lomberk, Fernandez-Zapico et al. 2005). Cell surface notch receptors, activated by transmembrane ligands of the Delta and Jagged families, expressed on neighbouring cells, direct cell fate decisions and promotes vascularisation of tumours when activated(Rehman and Wang 2006).

1.4.8 Hedgehog pathway

The Hedgehog pathway has a significant role in the development and growth of PDAC as well as the tumour microenvironment. The pathway includes the ligands, Sonic hedgehog, Indian hedgehog and desert hedgehog and their receptor proteins smoothened (smo) and patched (ptc). Research has shown abnormal expression of

components of this pathway in PDAC that affects tumour growth as well as induction of stromal desmoplasia in PDAC, which contributes to the chemoresistance of PDAC(Kayed, Kleeff et al. 2006; Bailey, Swanson et al. 2008; Yauch, Gould et al. 2008). Although, trials of the Hedgehog inhibitor Vismodegib have so far proved unsuccessful in sensitising PDAC to gemcitabine (Catenacci, Junttila *et al.* 2015)

1.4.9 Pathways in PDAC

Jones et al(Jones, Zhang *et al.* 2008) performed a global genomic analysis in human PDAC specimens to reveal the key pathways that are frequently mutated in pancreatic cancer shown in figure 1.2. This important work revealed 12 pathways that are commonly mutated, with some cancers having mutations in all of these and most displaying mutation of at least 9. The identification of theses core pathways has facilitated further understanding of the pathogenesis of PDAC as well as highlighting possible avenues for new therapy.

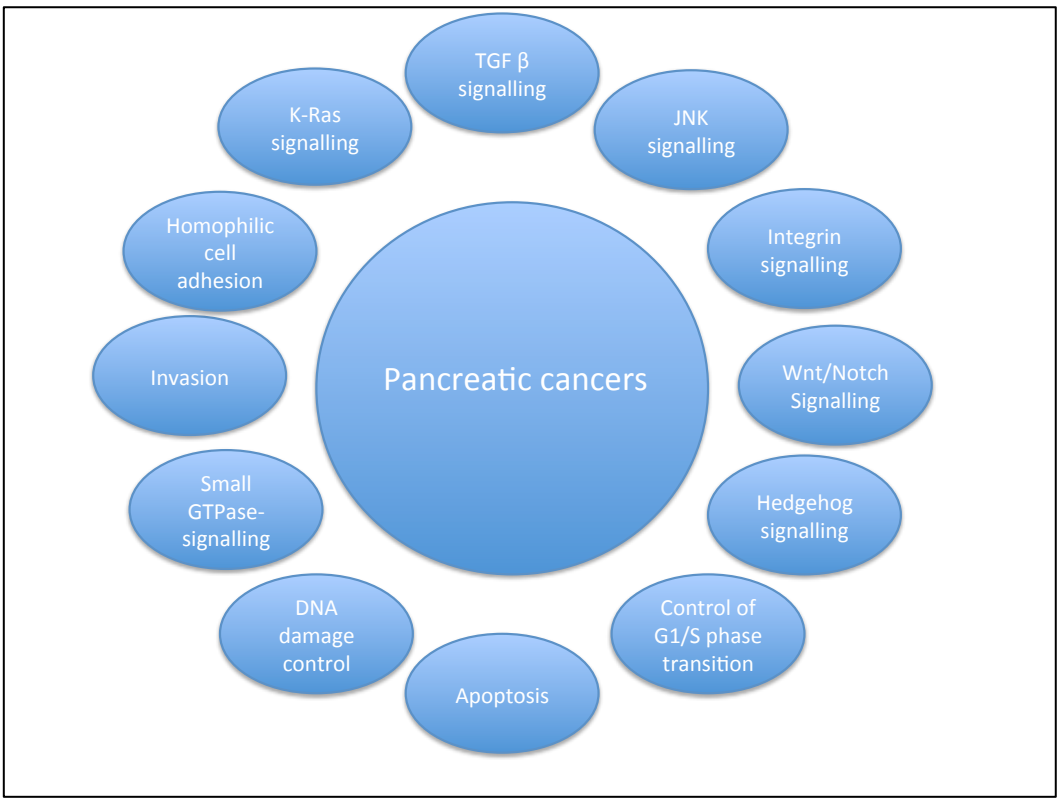


Figure 1.2 The 12 pathways and processes where component genes were genetically altered in the majority of pancreatic cancers identified on genomic analysis of 24 samples from Advanced PDAC (adapted from Jones et al).

1.4.10 Tumour microenvironment and stroma

One of the characteristics of pancreatic cancer is the formation of stroma surrounding the cancer termed a desmoplastic reaction(Chu, Kimmelman *et al.* 2007). This stroma surrounding pancreatic cancer cells is often poorly vascularised and contains a mixture of cellular and non-cellular components. Pancreatic stellate cells are a key component of stroma and have a critical role in the development and maintenance of the tumour microenvironment. When in a quiescent state these cells are characterised by the expression of desmin, glial fibrillary acidic proteins (GFAP) and vitamin A enriched intracellular fat droplets. When stimulated by growth factors such as TGF-β, platelet derived growth factor or fibroblast growth

factor the fat droplets disappear; the stellate cells then express alpha-smooth muscle actin and produce collagen fibres that make up a dense matrix surrounding the tumour(Mahadevan and Von Hoff 2007). This not only provides a mechanical barrier to the tumour but also results in a relatively hypoxic poorly vascularised tumour; both of these factors provide a therapeutic challenge(Erkan, Reiser-Erkan et al. 2009; Masamune and Shimosegawa 2009). Further features of these stromal cells have been associated with poor prognosis and resistance to treatment. Stromal cells are known to express PDGF receptor, vascular endothelial growth factor, chemokines, intergrins and hedgehog pathway components all of which contribute to the poor prognosis and relative therapeutic resistance of pancreatic cancers(Zhang, Erkan *et al.* 2007).

1.5 CURRENT THERAPY

1.5.1 Therapy in resectable disease

Surgical resection continues to be the only intervention for potential cure in 15-20% of patients(Alexakis, Halloran *et al.* 2004), the remainder presenting with unresectable disease. Even following a potentially curative resection, recurrence rates are high owing to residual macroscopic disease(Sperti, Pasquali *et al.* 1997). In an effort to improve survival, more radical approaches to surgical resections including extended lymphadenectomy and total pancreatectomy have been attempted, however, these have failed to prove any demonstrable survival

benefit(Fortner, Klimstra et al. 1996; Pedrazzoli, DiCarlo et al. 1998; Yeo, Cameron et al. 2002; Farnell, Pearson et al. 2005; Muller, Friess et al. 2007).

The basis of current therapies for PDAC involves targeting of DNA replication by inhibition of thymidylate synthase and incorporation of fluorouridine derivatives into nascent DNA strands. The agents that are used are 5-FU, capcetabine and gemcitabine and also have an effect on transcription and DNA methylation. These therapies are used in both the adjuvant and advanced disease setting based on previous trial evidence.

The ESPAC-1 trial was instrumental in providing clear evidence for the role of adjuvant chemotherapy and the lack of benefit from chemoradiation in improving survival following resection in pancreatic cancer(Neoptolemos, Stocken *et al.* 2004). The main finding from this study was a median survival of 20.1 months for those that received chemotherapy and 15.5 months in those that did not (hazard ratio 0.71, p=0.009). The results of CONKO-001 trial has added more weight to this argument which randomised patients to adjuvant gemcitabine or observation following surgery, patients that received chemotherapy had a improvement in overall median survival of 22.8 months compared to 20.2 months in the observation group (p=0.005)(Oettle, Post *et al.* 2007). The ESPAC-3(v2) trial has demonstrated that there is no significant survival difference between adjuvant gemcitabine and 5-

FU, and that the toxicity profiles of each would indicate that gemcitabine should be recommended above bolus 5FU/FA.(Neoptolemos, Stocken *et al.* 2010).

The use of fluorinated pyrimidine S-1 (an oral 5-flurouracil) as an adjuvant therapy in pancreatic cancer has been investigated and its use compared to gemcitabine. The JASPAC-01 trial was a randomised controlled study that enrolled 385 patients between 2007 and to 2010. The use of adjuvant gemcitabine was compared to S-1(Maeda, Boku *et al.* 2008). In 2012 interim results were published showing an overall 2-year survival of 53% for the gemcitabine group and 70% for the S-1 group(Fukutomi 2013). The disease free survival was also noted to be superior for S-1 at 49% compared to 29% in the gemcitabine arm. From this study the consideration of S-1 as standard adjuvant treatment was suggested and is now the case in Japan though this has not been adopted in the western world, this is due to the metabolic differences in the population treated and the observed gastrointestinal side effects being greater in Caucasian populations leading to lower tolerated doses of S-1 (Antoniou, Kountourakis *et al.* 2014).

In 2008 Boeck *et al* published a systematic review and meta-analysis of adjuvant chemotherapy in pancreatic cancer(Boeck, Ankerst *et al.* 2007). Five trials were identified suitable to be included in the meta-analysis (Bakkevold, Arnesjo et al. 1993; Takada, Amano et al. 2002; Neoptolemos, Stocken et al. 2004; Kosuge, Kiuchi et al. 2006; Oettle, Post et al. 2007) identifying a total of 482 treated patients and

469 controls. Median survival was increased by 3 months (95% CI 0.3–5.7 months, p = 0.03) in the adjuvant chemotherapy group. There was a small increase of 3.1% in 5-year survival in the adjuvant chemotherapy group compared with controls (95% CI –4.6 to 10.8%, p<0.05). The authors supported the use of adjuvant chemotherapy from this meta-analysis noting the statistically significant improvement in median survival.

The most recent meta-analysis into adjuvant therapy explored the overall survival in patients receiving gemcitabine (n=774) or 5-FU (n=876) versus observation (n=670). This study concluded adjuvant therapy with either agent conferred a survival benefit compared to observation alone (Hazard ratios 0.68, 95% CI: 0.44-1.07, and 0.62, 95% CI: 0.42-0.88, respectively). It was noted however that the grade 3-4 non haematological toxicity was far greater an issue with 5-FU than Gemcitabine(Liao, Chien *et al.* 2013).

Currently the results of ESPAC4 are set to change standard therapy after being released at the American Society of Clinical oncology. ESPAC 4 is a international randomised control trial of combination adjuvant therapy (gemcitabine and capecitabine) versus single agent adjuvant therapy (gemcitabine). Although the median survival benefit was modest in the combination group the overall 5-year survival for this group was double that of the gemcitabine alone cohort (28.8% vs.

16.3%). This study supports the notion that combining agents used in PDAC will offer the most promising therapeutic targets in the future(Neoptolemos 2016).

1.5.2 Therapy in advanced disease

In the advanced setting the initial standard of care was 5-FU. Evidence that 5-FU was superior to best supportive care was demonstrated by a meta-analysis of trials performed between 1970 and 2003(Fung, Takayama et al. 2003). Subsequently trial evidence supported the use of gemcitabine and its superior benefits in the advanced setting. Burris *et al* showed while having a modest survival advantage, gemcitabine gave better alleviation of symptoms than 5-FU (Burris, Moore *et al.* 1997). Sultana *et al* reviewed randomised control trials for advanced pancreatic cancer that included gemcitabine or 5-FU monotherapy, gemcitabine versus 5-FU or gemcitabine/5-FU combination therapy with other chemotherapy. This meta-analysis concluded that chemotherapy improved survival compared to best supportive care (hazard ratio =0.64, 95% CI 0.42-0.98), 5-FU based combination therapy was not superior to 5-FU alone, there was no significant survival difference between gemcitabine or 5-FU but a definite improvement in survival from gemcitabine combination therapy compared with gemcitabine alone (HR=0.91, 95% CI 0.85-0.97). This meat-analysis supported the concept that gemcitabine based combination therapy would provide the most improvements in survival in the advanced setting(Sultana, Smith *et al.* 2007).

Capecitabine (xeloda) was developed to be an orally available form of fluoropryrimidine chemotherapy. Cunningham *et al* were able to show that a combination of capecitabine and gemcitabine (GEM-CAP) performed better than gemcitabine alone with advanced disease, giving a genuine survival advantage in best-response rates (19.1% v 12.4%, p=0.03) and progression free survival (HR 0.78; CI, 0.66-0.93; x2 LR=8.1; p=0.004). There was even a trend to improved overall survival in the GEM-CAP group although this was not statistically significant (p=.0.8) (Cunningham, Chau *et al.* 2009).

Further combinations with gemcitabine have been explored without improved efficacy. Gemcitabine and oxaliplatin or gemcitabine with cisplatin have been investigated in phase III trials though no statistically significant improvements in survival have been noted(Louvet, Labianca et al. 2005; Colucci, Labianca et al. 2010).

A regimen referred to as GTX (docetaxel and capecitabine in addition to gemcitabine) has demonstrated some activity in the advanced disease setting though with a degree of toxicity. Phase II trials showed median survival of 25 and 11 months for patients with locally advanced and metastatic disease respectively. Though not standard first line therapy this combination could be considered for patients with a good performance status seeking combination therapy(De Jesus-Acosta, Oliver et al. 2012).

Further combinations of gemcitabine with targeted therapies have yielded only small (gemcitabine plus erlotinib) or no survival benefits (cetuximab or bevacizumab plus) with notably increased toxicities therefore currently these combinations are not used in standard therapy and offer no real benefit over gemcitabine alone(Moore, Goldstein et al. 2007; Kindler, Niedzwiecki et al. 2010; Philip, Benedetti et al. 2010).

Recently there has been investigation of the FOLFIRINOX regimen in advanced pancreatic cancer (consisting of oxaliplatin, irinotecan, fluorouracil and leucovorin)(Conroy, Desseigne *et al.* 2011). In this study the overall survival was significantly better in the FOLFIRINOX group at 11.1 months compared to 6.8 months in the gemcitabine group (HR 0.57, 95% CI 0.45-0.73, $p<0.001$). However more adverse event were noted in the FOLFIRINOX arm which had a significant effect on quality of life scores making the combination questionable in the setting of advanced pancreatic cancer owing to unacceptable toxicity in the absence of a good performance status.

1.5.3 Neoadjvant therapy

The use of neoadjuvant therapy to improve the outcome for these patients by down-staging disease, increasing resection rates and R0 resection margins(Gillen, Schuster *et al.* 2010) has been evaluated in small studies and not in a successful phase III randomized trial so far. Some studies suggest an increase in resection rate

by around a third(Greer, Pipas et al. 2008; Gillen, Schuster et al. 2010). A number of studies demonstrate an increase in survival rate comparable with resectable disease(Gillen, Schuster et al. 2010; Stokes, Nolan et al. 2011; Takahashi, Kinoshita et al. 2011; Motoi, Ishida et al. 2013).

Neoadjuvant therapy may offer a survival benefit to those with borderline resectable disease by downstaging tumours, improving resection rates and negative margins, however role of neoadjuvant therapy in BR disease has been investigated thus far in small phase I/II trials(Brown, Siripurapu et al. 2008; Landry, Catalano et al. 2010; McClaine, Lowy et al. 2010; Patel, Hoffe et al. 2011; Sahora, Kuehrer et al. 2011; Barugola, Partelli et al. 2012). It appears that neoadjuvant therapy may have no benefit or even a detrimental effect in those initially classified resectable, but in borderline resectable groups improvements in survival of up to a third are suggested. Studies to date consistently demonstrate an improved survival in borderlines receiving neoadjuvant therapy over non-resectable cases and even comparable survival to resectable cases which was supported in recent meta-analyses(Gillen, Schuster et al. 2010; Assifi, Lu et al. 2011; Laurence, Tran et al. 2011; Andriulli, Festa et al. 2012). A recent prospective study of a small group of patients by Lee *et al* suggested curative resection is possible in patients with borderline resectable disease who undergo neoadjuvant therapy (Lee, Kim *et al.* 2012).

Further potential benefits of neoadjuvant therapy include possible increased efficacy of chemoradiation and chemotherapy to a well-vascularized and

oxygenated tumour as well as identifying patients who have aggressive disease as in this group of patients it has been demonstrated that surgery after neoadjuvant therapy offers no real improvement in survival(Evans, Rich et al. 1992; Pisters, Abbruzzese et al. 1998).

1.5.4 Chemoresistance in PDAC

Despite the demonstrated survival benefits recurrence rates in resected disease remain high and in all disease settings long-term survival remains poor due in part to chemoresistance. Cancer cells become resistant to therapy by a number of hypothesised means: by exclusion of drugs from the cancer cells, by changes in enzymes metabolising drug or by becoming resistant to apoptosis and cellular stress. Further increases in survival should be possible by targeting these areas that lead to resistance or targeting multiple pathways in combination with standard therapy.

The lack of efficacy of current treatment may be explained by inadequate drug delivery to the cancer cells leading to ineffective concentrations. This may be attributable to the tumour microenvironment consisting of a stromal extracellular matrix protecting cancer cells or transport of fluoropyrimidines across the plasma membrane of cancer cells. It may be these agents are not transported into resistant cells or moved out more rapidly. There are two types of nucleoside transporters that transport gemcitabine across the plasma membrane, namely equilabratitive (eNTs) and concentrative (cNTs) and there has been investigation into these

transporters and their relationship to gemcitabine resistance although cell line data has been inconclusive. Gemcitabine is transported by hENT1, hENT2 and hCNT1. Once gemcitabine enters a cell via eNTs it reaches equilibrium between the intracellular and extracellular compartments, cNTs increase the concentration of the drug intracellularly by accumulation against the concentration gradient. Marechal *et al* observed that either high expression of eNT1 or cNT resulted in the best overall survival(Marechal, Mackey *et al.* 2009) and the high expression of eNT1 and the positive effect on survival was again supported in the RTOG-9704 trial(Farrell, Elsaleh *et al.* 2009). However contrary finding were reported by Tsujie *et al* who demonstrated a direct liner relationship between the levels of eNT1 mRNA and the IC50 for gemcitabine and 5-FU(Tsujie, Nakamori *et al.* 2007). Interestingly in the research setting pancreatic cancer cell lines are sensitive to gemcitabine and 5-FU regardless of eNT status when adequate concentrations of drug are delivered to the cell membrane which goes some way to explaining why xenograft models show a good response to these agents.(Olive, Jacobetz *et al.* 2009). Conversely in transgenic mouse models pancreatic cancer shows resistance to gemcitabine similar to what is observed in the clinical setting ad this seems to be a consequence of the inability of drug to penetrate the tumour stroma(Olive, Jacobetz et al. 2009; Grippo and Tuveson 2010; Neesse, Michl et al. 2011) an issue not just for these agents but many agents used in pancreatic cancer.

Owing to the observed relative chemoresistance of pancreatic cancer to current therapy and the knowledge of the underlying molecular biology in PDAC there has

been investigation into novel therapies both as monotherapies and in combination with gemcitabine.

1.6 NOVEL THERAPY IN PDAC

As previously discussed Kras mutations are the single most common genetic change observed in PDAC(Almoguera, Shibata *et al.* 1988). The effects of farnesylation inhibitors has been investigated owing to the fact that the ras protein undergoes farnesylation in order to adhere it to the plasma membrane. In early xenograft models promising effects were observed. Manumycin, one such agent, inhibited growth in a xenograft model of pancreatic cancer without toxicity(Ito, Kawata *et al.* 1996). SCH 66336, a small molecule farnesyl protein transferase inhibitor, was investigated in tumour xenograft models of colon, lung, pancreas, prostate and bladder cancers. In these, potent oral activity was observed leading to a reduction in tumour burden. The investigators went on to a transgenic mouse model and observed significant tumour regression in a therapeutic model associated with increased apoptosis and decreased DNA synthesis in tumours(Liu, Bryant *et al.* 1998). Unfortunately, when entered into clinical trials these agents have so far been ineffective(Macdonald, McCoy *et al.* 2005). This may be due to the fact that in cells treated with the agents Kras is geranylgeranylated thus allowing membrane attachment. Subsequently newer agents are being developed and tested in mouse models and phase I/II trials(Campbell, Boufaied *et al.* 2010).

Another common change seen in pancreatic cancer is the expression of epidermal growth factor and there are readily available inhibitors such as elrotinib and cetuximab. In retrospective studies a significantly reduced median survival has been observed for patients expressing high levels of epidermal growth factors providing a biological rationale for treatment(Yamanaka, Friess *et al.* 1993). The SWOG S0205 study reported finding on gemcitabine plus cetuximab versus gemcitabine alone at ASCO 2007 (look at (Philip, Benedetti *et al.* 2010)). Patients with locally advanced or metastatic PDAC were enrolled into each arm. In the primary endpoint of overall survival there was no significant benefit to combination therapy (HR 1.09, 95% CI 0.93-1.27, p=0.14) nor in secondary endpoints of Progression Free Survival (PFS) and response (philip 2007). In clinical trails with pancreatic cancer erlotinib in combination with gemcitabine showed a modest survival benefit in the setting of advanced cancer(Moore, Goldstein *et al.* 2007).

A potential target in pancreatic cancer is that of VEGF receptor inhibitors. One such inhibitor is avastin (bevacizumab), which was studied in a phase II trial in combination with ertloinib and gemcitabine. The primary endpoint of 6 months survival was not reached for most patients but stable disease was achieved for more than 2 cycles of treatment for 22.2% of the patients(Ko, Venook *et al.* 2010). A phase III trial into bevacizumab in combination with gemcitabine and erlotinib was conducted. Patients with metastatic PDAC were assigned to gemcitabine plus erlotinib and bevacizumab or placebo with the primary endpoint of overall survival. In the group that received all active agents the OS was not significantly improved compared to gem, erlotinib plus placebo but the therapy was well tolerated and the

secondary endpoint of progression free survival was improved (HR 0.73; CI 95%0.61-0.86, p=0.0002)(Van Cutsem, Vervenne *et al.* 2009).

As previously discussed the relative resistance to current therapy may be the deficiency in apoptotic features in cancer cells. Platinum based therapies have been shown to strongly stimulate apoptosis(Saif and Kim 2007). Studies of platinum agents in pancreatic cancer have shown significant benefit when these are used in combination with gemcitabine. The combination of cisplatin and gemcitabine has been shown synergistic *in vitro* and in a phase II study improvements where observed in disease free survival and response although there was no significant difference in overall survival when compared to gemcitabine monotherapy(Colucci, Giuliani *et al.* 2002). Another such phase II study of a cisplatin analog, carboplatin, combined with gemcitabine showed an encouraging 1-year survival of 28%(Xiros, Papacostas *et al.* 2005). Combinations with both gemcitabine and 5-FU have been investigated with oxaliplatin and shown synergy in the pre-clinical setting. In patients with pancreatic cancer refractory to gemcitabine monotherapy, GEMOX has shown promising survival benefit(Demols, Peeters *et al.* 2006). A similar suggestion of improved survival in gemcitabine refractory tumours has been shown with the combination of oxaliplatin and 5-FU, FOLFOX(Yoo, Hwang *et al.* 2009). In a meta-analysis of novel combination therapy in PDAC, platinum agent based combinations showed the highest response rates overall(Sultana, Ghaneh *et al.* 2008).

Targeting proteins that feature in the tumour stroma may also provide another therapeutic avenue. A study by Olive *et al* investigated a mouse model of pancreatic cancer targeting the stromal hedgehog pathway. They used KPC mice, which conditionally express mutant Kras and p53 in PDAC and develop tumours that resemble human PDAC in both molecular and pathophysiological features. In the study the tumours developed were poorly vascularised and delivery and efficacy of gemcitabine also poor, in line with clinical observations. They went on to inhibit the hedgehog pathway using IPI-926, a derivative of cyclopamine that potently inhibits Smo, this resulted in increased tumour vascularisation and therefore the delivery of chemotherapeutic agents to the tumour(Olive, Jacobetz *et al.* 2009). However, a clinical trial of hedgehog inhibition showed no improvement over gemcitabine alone (Catenacci, Junttila et al. 2015)

As indicted by Hanahan and Weinberg unlimited proliferation is a trait intrinsic to the survival of cancer cells(Hanahan and Weinberg 2011). In humans there are cell-autonomous features that limit replicative potential and induce cellular senescence. Cell senescence serves as a barrier to limitless proliferation and is dependent on the p53 and RB tumour suppressor pathways. With mutations in these pathways cancer cells overcome checkpoints and the genomic instability experienced can be attributed to the attrition of telomeres. In contrast to normal cells, cancer cells show telomersase activity and inhibition can lead to rapid telomere shortening and apoptosis, providing a rationale for therapy. A number of studies have demonstrated up regulation of telomerase in pancreatic cancer(Hiyama, Kodama et

al. 1997; Suehara, Mizumoto et al. 1997). There has been investigation into the use if immune technology to target and ameliorate telomerase positive cells. GV1001 has been shown to elicit T cell response in lung cancer without bone marrow suppression(Brunsvig, Aamdal *et al.* 2006) and there was a phase I/II study conducted in unresectable pancreatic cancer to investigate safety and tolerability(Bernhardt, Gjertsen *et al.* 2006). In this study a significant immune response was observed in 75% of the group receiving therapy. The vaccine was well tolerated with no significant adverse effects. In this study overall survival was not a primary endpoint though a significant correlation was observed with median survival of 8.6 months in patients receiving a moderate dose compared to 4.5 months in those receiving a low or high dose. After 300 days all surviving patients had demonstrated an immune response to the vaccine. The TeloVac trial is the largest study of GV1001 in pancreatic cancer to date and examined gemcitabine and capecitabine therapy with concurrent or sequential GV1001 treatment in patients with locally advance or metastatic disease. This showed no significant survival benefit with the vaccine (Middleton, Silcocks et al. 2014).

MEK inhibition has also been investigated in pancreatic cancer. Mitogen-activated extracellular signal regulated kinase is a crucial component of the RAS-RAF-MEK-extracellular signal regulated kinase (ERK) that has a role in several cellular processes and known to be aberrant in a number of cancers including pancreas, breast, lung and colon(Hoshino, Chatani *et al.* 1999). A MEK1/2 inhibitor, CI-1040, demonstrated anti-proliferative activity in a xenograft model of pancreatic

cancer(Allen, Sebolt-Leopold *et al.* 2003). In a phase I study of patients with advanced malignancies including pancreatic cancer it was well tolerated(Lorusso, Adjei *et al.* 2005). In this phase I study it was noted that one of the patients with pancreatic cancer demonstrated a partial response but on progression to a phase II study no significant antitumor activity was seen and the study abandoned.

Gastrin inhibition has also been investigated in pancreatic cancer, as expression of cholecystokinin (CCK)/gastrin receptor and the gastrin precursor, progastrin, have been observed frequently in pancreatic tumours(Caplin, Savage *et al.* 2000). Gastrin stimulates the proliferation of pancreatic cancer that can be inhibited by antisense oligonucleotides to gastrin(Smith, Fantaskey et al. 1995; Smith, Verderame et al. 1999). Gastrazole, a gastrin receptor antagonist was studied in two trials in pancreatic cancer compared to placebo first then with 5-FU. Compared to placebo median survival was significantly improved with gastrazole (7.9 months vs. 4.5 months) but there was no significant difference when compared to 5-FU(Chau, Cunningham *et al.* 2006).

Targeting the enzymes that allow cells to degrade their surrounding matrix therefore facilitating metastasis has provided another area of investigation in pancreatic cancer. Matrix Metalloproteinase (MMPs) are proteolytic enzymes that are involved in the degradation of the extracellular matrix and an association between MMP expression and tumour progression has been described(Chambers

and Matrisian 1997). In pancreatic cancer MMP-2,-7,-8,-9 and -11 are overexpressed and high MMP-7 levels correlate with a poor survival(Jones, Humphreys *et al.* 2004) . In preclinical models MMP inhibitors have shown efficacy. SC44463, a novel MMP inhibitor prevented metastases in an early mouse model(Reich, Thompson *et al.* 1988). A further MMP inhibitor Batimastat was investigated in a orthotopic murine model of pancreatic cancer and demonstrated reduced metastasis and significantly prolonged overall survival as a monotherapy and in combination with gemcitabine(Zervox, Franz *et al.* 2000). In clinical trials however, results were not as promising. A trial of patients with advanced pancreatic cancer treated with Tanomastat was terminated early as patients experience a significantly worse survival than those receiving placebo(Moore, Hamm *et al.* 2003). Marimastat has been extensively investigated in human trials of pancreatic cancer. A phase II study showed an acceptable toxicity profile and some symptomatic improvement in patients(Evans, Stark *et al.* 2001) and as a result of this, two randomised phase III trials were conducting comparing gemcitabine and Marimastat in patients with unresectable PDAC. In both of these no significant survival benefit was seen and there was no significant improvement in combination therapy with gemcitabine when compared to gemcitabine alone(Bramhall, Rosemurgy et al. 2001; Bramhall, Schulz et al. 2002). It may be that this agent merits further investigation as study design was flawed: patients enrolled had advanced disease and the evidence from preclinical data in reducing progression suggests that patients with early disease may derive the most benefit from these types of agents.

Another line of investigation into potential novel therapy is that of COX inhibition. COX-2 plays a principal role in the metabolism of prostaglandins therefore mediating resistance to apoptosis, adhesion, motility, immunosuppression and angiogenesis (Trifan and Hla 2003)and has been shown to be expressed in up to 90% of pancreatic cancers(Okami, Yamamoto *et al.* 1999). Results on the efficacy of these agents in pancreatic cancer have been variable. In a phase I study of celecoxib with a gemcitabine-radiation regimen increased efficacy as well as significant toxicity was observed(Crane, Mason *et al.* 2003). In another study by Milella *et al* no demonstrable clinical benefit was derived from the addition of celecoxib to 5-FU therapy in gemcitabine refractory disease(Milella, Gelibter *et al.* 2004). In a phase II trial in advanced patients of combination therapy with gemcitabine and celecoxib median survival was 6.2 months and the toxicity profile was acceptable. A further phase II trial found a median survival of 9.1 months with this combination, also in advanced disease (El-Rayes, Zalupski *et al.* 2005). Though the data thus far is conflicting the promising results that have been observed suggests further investigation of these agents in pancreatic cancer is warranted.

Inhibitors of PI3K/AKT pathway have been suggested as potential agents. It has been demonstrated that inhibition of the AKT pathway in pancreatic cancer induces apoptosis and restores gemcitabine chemosensitivity *in vitro* offering an interesting therapeutic strategy(Ng, Tsao et al. 2000; Bondar, Sweeney-Gotsch et al. 2002). Targeting downstream of AKT has been investigated using mTOR inhibition. In

preclinical studies CCI-779, an mTOR inhibitor supressed proliferation in pancreatic cancer cell lines and induced apoptosis(Asano, Yao *et al.* 2005).

Most of these therapies have as a virtue the specificity of the agent for a particular target (from KRAS to ribonucleotide reductase via AKT and MMP-7). It has been a struggle to achieve such specificity and success has been marginal. Perhaps it is worth considering whether this apparent virtue is truly desirable.

As previously mentioned Jones *et al* identified 12 common pathways which were commonly affected in advanced cancer, but with multiple possible mutations and considerable heterogeneity. Clearly this underpins the complex genetic anomalies that exist in PDAC and supports the concept that targeting single genes or proteins will likely be ineffective. Therapy that targets multiple pathways at different levels in combination with standard therapy may offer the most chance of making significant differences to survival of patients. A protein that appears on multiple pathways commonly mutated in PDAC is Heat Shock Protein (HSP) 90 and may offer a possible target for future therapy. Genetic mutations that arise in pancreatic cancer often have effect on crucial checkpoints within the cell cycle and CDKs, being pivotal to cell cycle regulation, could offer another target for inhibition in pancreatic cancer. Targeting HSP90 and CDK inhibition could offer future promise for therapy in PDAC.

The key to the future of chemotherapy in pancreatic cancer will be the identification of novel and effective agents, better biomarker technology underpinned by translational research, which will inform the design of future trials. Ultimately this will ensure that patients will be able to receive targeted therapy to achieve the most benefit.

1.7 CYCLIN DEPENDANT KINASES AND CANCER

The replication of DNA and mitosis are reliant on the activity of cyclin-dependant protein kinase (CDK) enzymes. CDK's are heterodimers of a catalytic subunit with a cyclin subunit whose activity governs cell cycle regulation.

1.7.1 Structure

Protein kinases have a tertiary structure consisting of a small amino-terminal lobe and a larger carboxy-terminal lobe (figure 1.3). ATP fits between these lobes with phosphates directed outwards towards where the protein substrate binds. There is then transfer of the terminal γ-phosphate of ATP to hydroxyl residues. CDKs are inactive in the absence of cyclin via two modifications when compared to other protein kinases. Firstly, in CDKs a T-loop or activation loop arises from the carboxy-terminal lobe thereby blocking the binding of protein substrate at the entrance to the active cleft site. Secondly there are several important amino-acid side chains in the inactive CDK that are incorrectly positioned so that the ATP phosphates are not well oriented for the kinase reaction. As a result of these two factors CDK activation

requires structural change in the active site. This is facilitated by interaction of the a1 helix of the upper lobe with a cyclin, this results in reorientation of the residues that interact with the phosphates of ATP. The reconfiguration is also facilitated by the small L12 helix that changes structure to become a beta strand during cyclin binding.

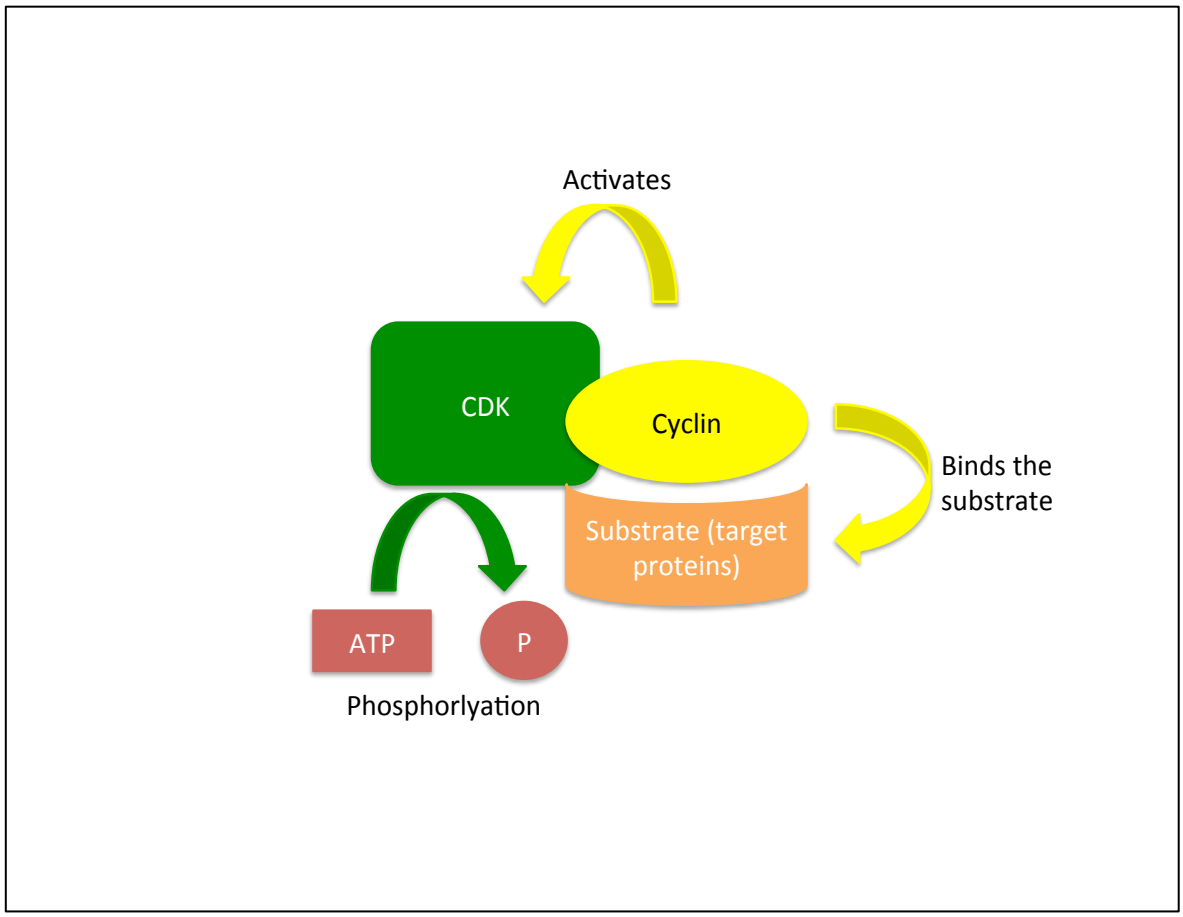


Figure 1.3 The structure and conformity of a cyclin dependant kinase molecule.

1.7.2 The cell cycle

Progression through the cell cycle involves changing abundance of specific cyclins with subsequent changes in the nature of CDK activity.

The cell cycle is an ordered sequence of events resulting in cell growth and division into two daughter cells. During S phase chromosome replication takes place and during M phase (mitosis) segregation of the replicated chromosomes occurs. There are two Gap phases: the phase prior to S is G1 and prior to M is G2. The double helix structure of DNA unwinds in S phase producing two templates used for the synthesis of two new DNA strands. In G2 a bipolar spindle is formed and the DNA helix molecules making up each replicated chromosome condense and during metaphase move to the centre of the spindle and orientate towards opposite poles, to be segregated at cell division. The ordered cycling that occurs is dependent on CDKs and their formation of CDK-cyclin complexes and each CDK acts at a specific point in the cell cycle. CDK4 and 6 interact with cyclin D, CDK2 with cyclin E, allowing passage through the G1 restriction point into S phase. Phosphorylation of Rb via cyclin D-CDK4/6 late in G1 phase results in the components necessary for DNA replication. Cyclin E-CDK2 leads to further phosphorylation of pRb that facilitates entry to S phase. Cyclin A-CDK2 (and then CDK1) prevents re-initiation of DNA synthesis. Completion of DNA replication in S phase is followed by G2, there are 3 checkpoints that need to be passed in order to begin segregation of chromosomes at anaphase. First a checkpoint to ensure that there is no DNA damage (the DNA checkpoint), then a checkpoint to ensure centrosome segregation and correct spindle formation (the CHFR checkpoint) and then a checkpoint to ensure that the chromosomes are correctly orientated at the metaphase plate (the spindle or tension checkpoint). These are controlled by cyclin B-CDK1, gradual

increase in activity allowing metaphase to be achieved and rapid decline allowing passage into anaphase. CDC25 phosphatase dephosphorylates cyclin B-CDK1 facilitating its activation during metaphase, subsequently cyclin B is degraded by the proteasome as a result of ubiquitination by the anaphase-promoting complex (APC). A feedback loop results in phosphorylation of APC by cyclin B-CDK1 that is necessary for cyclin B proteolysis and subsequent transition into anaphase. The cyclins and their CDKs with points of action in the cell cycle are summarised in table 1.7 and figure 1.4.

Phase	Cyclin	CDK
G1	D, E	CDK 4, 6, 2
S	A, E	CDK 2
G2	A	CDK 2, 1
M	B	CDK 1

Table 1.7 Table showing cyclins and CDK's and their points of action in the cell cycle.

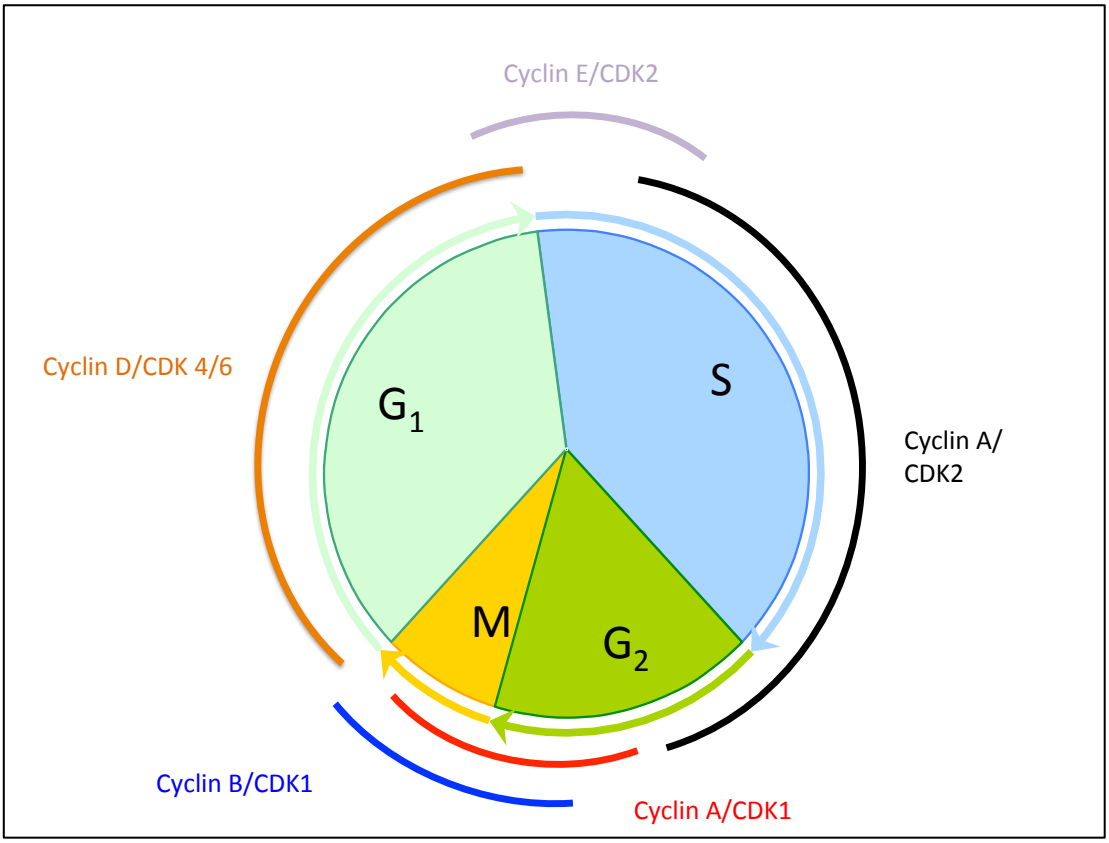


Figure 1.4 The cell cycle with CDK/cyclin complexes at points where they are known to act, adapted from Suryadinata et al ((Suryadinata, Sadowski et al. 2010)

CDKs were identified as enzymes that control cell cycle events, though members of the CDK family are involved in other cellular processes. In human cells CDK 1, 2, 4, and 6 are directly involved in cell cycle control and CDK 7 works indirectly by acting as a CDK-activating kinase (CAK) phosphorylating other CDKs. CDK 7, 8 and 9 are involved in gene transcription and CDK 5 in the differentiation of nerve cells.

1.7.3 CDKs and Cancer

Cancer cells, by definition, are prone to inappropriate cell division and the activity of CDKs and cyclins are frequently deranged in cancer. This may be due to

amplification of genes encoding for specific CDKs or cyclins, but it is more common to have loss of expression of a CDK inhibitor such as $p16^{INK4a}$(Malumbres and Barbacid 2001). Proteins involved in CDK regulation and downstream substrates have also been linked to tumour development. Common deregulated cyclins, CDKs and endogenous CDK inhibitors are summarised in table 1.8 with the cancers they are associated with.

Genetic alterations in CDKs themselves are not that common but have been observed in some human cancers. Mutations and amplifications in CDK4 have been noted in some tumours including melanomas, breast carcinomas, gliomas and sarcomas (He, Allen et al. 1994; Wolfel, Hauer et al. 1995; Kanoe, Nakayama et al. 1998; An, Beckmann et al. 1999). CDK6 has been found amplified in lung tumours(Zhao, Weir *et al.* 2005) and translocated in melanoma(Okamoto, Pirker *et al.* 2005).

Deregulated expression of cyclins is a feature in human cancer, cyclin D1 is of particular note and has been identified in haematological malignancies as well as breast cancers, the overexpression of cyclin D1 is involved in aberrant Rb pathways in cancer (Barbareschi, Pelosio et al. 1997; Malumbres and Barbacid 2001). Overexpression of cyclin E is seen in many tumour types and has been evaluated as a potential prognostic marker owing to its correlation with clinical response(Hwang and Clurman 2005).

Relationships between CDK's cell cycle regulation and tumour suppressor genes are critical in cancer. Aberrant pRb signalling pathways are common in the majority of human cancers resulting in unchecked entry into S phase. Overexpression of cyclin D1 or deletion of $p16^{INK4A}$ activates the pRb pathway in this manner. p53 is an essential regulator of the G1 and G2 checkpoints and arrests growth through induction of p21 (a CDK inhibitor) and G2 arrest by promoting transcription of GADD45 that inhibits cyclin B-CDK1 activation.

In pancreatic cancer a number of genetic abnormalities have been identified including those that rely on or are closely involved in cyclins and CDKs. This includes inactivation of p16, a endogenous CDK inhibitor. Cyclin D synthesis and coupling with CDK partners is stimulated by growth factor signalling, under normal conditions p16 normally prevents cell cycle progression by binding with CDK and preventing the CDK phosphorylation of Rb. The drive into S phase is finally permitted when p16 is sequestered by the production of cyclin D-CDK complexes allowing Rb phosphorylation and deactivation. This pathway (p16/Cyclin D/Rb)is critical in tumorigenesis(Sherr 1996). Mutation of p16 are frequent in pancreatic cancers the majority being nonsense mutations in a single allele with silencing by methylation of the other allele, the second most common form of inactivation is homozygous deletions. p15 activity has also been investigated in pancreatic cancer and its inactivation been found in up to 60% of patients(Goggins, Shekher *et al.* 1998). This alteration in p15 activity may be in part responsible for the resistance to

TGF-β inhibitory effects that pancreatic cancer displays (TGF-β inhibits epithelial cell growth)(Villanueva, Garcia *et al.* 1998). Under normal conditions the downstream cell cycle inhibitor p15 forms a complex with CDK4 and 6 resulting in cell cycle arrest which is one of the mechanisms by which TGF-β inhibits growth(Hannon and Beach 1994).

Kras mutations are frequent and almost universal in pancreatic adenocarcinoma and activity of ras appears central to pancreatic tumorigenesis. The expression of cyclin D1 is regulated by ras, the elevated cyclin D1 levels along with the aforementioned disruption of the Rb/p16 pathway allows the maintenance of proliferative drivers in pancreatic cancer(Ji, Tsou et al. 2009).

Target	Oncogenic change	Associated tumours
a) Cyclin D1	Gene amplification Overexpression translocation	40-80% breast carcinomas 70% familial polyposis 50% B cell lymphoma 35% head and neck carcinomas 25-50% oesophageal carcinomas 25% bladder tumours
Cyclin E	Overexpression Gene amplification	90% colorectal carcinomas 30-80% breast carcinomas 70% prostate carcinomas 18% ovarian carcinomas
Cyclin E2	Overexpression	Breast carcinoma Small-cell lung carcinoma Cervical carcinoma
Cyclin B1	Overexpression	90% colorectal carcinoma
Cyclin A	Amplification or overexpression	Hepatocelluar carcinoma
CDK2	Overexpression	Colorectal carcinomas
CDK4	Amplification	Sarcomas, gliomas
b) p15	Deletions	pancreatic
p16	Mutation Deletion Epigenetic	Pancreatic carcinoma Melanoma Gilomas Bladder Head and neck Non small cell lung Lymphoma/leukaemia
p21	Mutation Downregulation Mislocalisation	Oral Oesophageal Breast
p27	Mutations/deletion rare downregulation	Breast Colon prostate

Table 1.8 Table showing a) deregulated cyclins and CDKs and their associated tumours b) deregulated endogenous CDK inhibitors and associated tumours(Benson, Kaye et al. 2005)

1.7.4 CDK inhibition in cancer

The role of aberrant CDK activity and potential inhibition has been investigated in many human cancers.

Flavopiridol is broad-spectrum CDK inhibitors with proven activity *in vitro* against CDK1, 2, 4 and 6 and cell effects including inhibition of growth and proliferation, induction of apoptosis and inhibition of angiogenesis. In mouse studies poor oral bioavailability was evident and subsequent parenteral dosing did result in some GI and marrow suppressive toxic effects but there was significant antitumor effects and significantly synergistic effects with other chemotherapeutics. Phase I clinical trials have been completed with flavopiridol as a monotherapy in refractory tumours with GI dose limiting toxicity being the main issue. Phase II trials have yielded significant effects in non-small cell lung cancer that has resulted in a phase II trial comparing flavopiridol to standard therapy(Shapiro 2004).

Yu *et al* performed a range of experiments concerning cyclin D-CDK4 activity in Breast cancer. Initially they demonstrated that mice lacking in cyclin D1 are resistant to breast cancers triggered by Erb-2 oncogene(Yu, Geng *et al.* 2001) then developed this hypothesis proposing that the ability of cyclin D to activate CDK4 underpinned the oncogenic potential of Erb-2-driven breast neoplasia. This was tested by crossing CDK4-null mice with a transgenic strain in which the expression

of Erb-2 gene was driven by a mammary specific mouse mammary tumour virus enhancer. The result of this was complete protection against breast cancers(Yu, Sicinska *et al.* 2006). Interestingly the normal physiological development of breast tissue in the CDK4-null mice was unimpeded suggesting the requirement for CDK4 activity in neoplasia but not normal mammary development. These conclusions were supported by Landis *et al* (Landis, Pawlyk *et al.* 2006) again supporting the concept that CDK's offer an attractive target in cancer therapy.

Work on CDK2 targeting has not provided such promising results. Testu *et al* investigated CDK2 inhibition and noted its activity is indispensible for both colonic neoplasia and other cancer cell types including cervical and osteosarcoma(Tetsu and McCormick 2003). Despite this there have been reports of successful targeting of CDK2 in other tumour types. Du *et al* demonstrated CDK2 activity was critical for melanoma cell proliferation demonstrating that specific CDKs may offer therapeutic targets in specific tumour types(Du, Widlund *et al.* 2004).

Novel CDK inhibitors have also been investigated in PDAC. Brasca *et al* reported on a novel CDK inhibitor PHA-793887 and its effects in a xenograft model of pancreatic cancer using the tumour line Bx-PC3. The agent was well tolerated and resulted in significant tumour growth inhibition (Brasca, Albanese *et al.* 2010).

The effects of the CDK inhibitor Dinaciclib (SCH727965; MK-7965) in combination with an AKT inhibitor (MK-2206) were investigated in orthotopic and subcutaneous xenograft models of pancreatic cancer. In this study tumour growth and metastases were blocked in the models used and several complete responses were noted in this combination, a phase I trial for pancreatic cancer is now ongoing (Hu, Dadon *et al.* 2015).

1.7.5 AT7519- A novel CDK inhibitor

AT7519 is a general CDK inhibitor developed by Astexs pharmaceuticals (Cambridge, UK). This compound was developed using fragment-based X-ray crystallographic screening. Fragments are low-molecular weight compounds with low binding affinities and screening using X-ray crystallography yields small libraries of compounds that have much greater probability of yielding complimentary fragments and targets(Hartshorn, Murray *et al.* 2005). Astexs developed a panel of molecules and investigated pharmacokinetic properties leading to the identification of AT7519, its chemical structure shown in figure 1.5 (Wyatt, Woodhead *et al.* 2008).

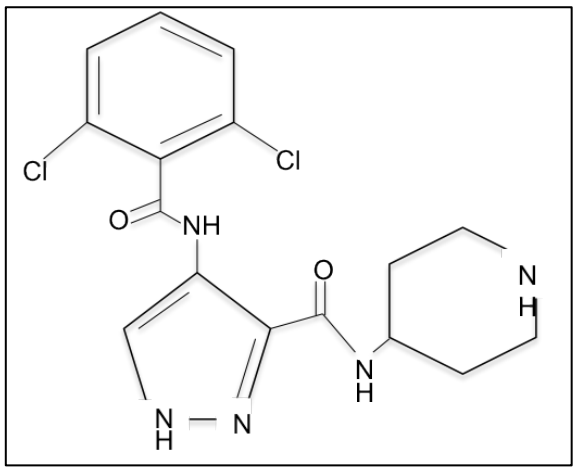


Figure 1.5 The structure of AT7519 adapted from Squires et al (Squires, Feltell et al. 2009).

AT7519 is a potent inhibitor of CDK1,2, 4 , 6 and 9 and also inhibits CDK 3 and 7 to a lesser degree. Initial *in vitro* experiments across a range of cancer cell lines confirmed the antiproliferative (table 1.9) activity of AT7519 and TUNEL staining assays and colony formation confirmed induction of apoptosis in colonic and ovarian cancer cell lines, interestingly an exposure equivalent or greater than one cell cycle was required for induction of apoptosis suggesting that AT7519 needed to be present during certain points in the cell cycle(Squires, Feltell *et al.* 2009). In this initial investigation a xenograft model using HCT116 (a colonic cancer cell line) was carried out that showed delayed tumour growth and tumour regression in an advanced cancer model compared to controls ($p<0.05$). CDK2 substrates were also tested from this xenograft model, namely the phosphorylation of NPM and Rb and were shown to decrease after certain time points post dose.

Origin	Cell Line	AT7519 IC50 (nM)
Colon Carcinoma	HCT116	82
	HT-29	170
	SW620	940
Ovarian carcinoma	A2789	350
	SK-OV-3	400
Lung Carcinoma	A549	310
	NCI-H69	330
	NCI-H596	690
	NCI-H82	230
Breast Carcinoma	MCF-7	40
	BT-20	320
	MDA-MB-468	340
	SK-BR3	140
	HCC1937	460
Uterine sarcoma	MESSA	660
Leukaemia	HL60	230
	K562	310
	MOLT-4	310
	F36-P	390
	Kasumi-1	250
	MOLM-13	170
	MV4-11	170
	PL-21	160
Lymphoma	Granta-519	160
	JEKO-1	670
	Ramos	720
Fibroblast	MRC-5	980
	MRC-5 (nonproliferating)	>10,000

Table 1.9 The anti-proliferative activity of AT7519 in a range of human cancer cell lines adapted from Squires et al (Squires, Feltell et al. 2009).

Since its initial description AT7519 has been investigated in the preclinical setting in a range of malignancies, efficacy has also been demonstrated in multiple myeloma, leukaemia and neuroblastoma cell lines(Santo, Vallet *et al.* 2010) (Squires, Cooke et al. 2010; Dolman, Poon et al. 2015).

Phase I studies have characterised the toxicity profile of this agent and shown initial promise regarding efficacy. Patients with refractory solid organ tumours or non-Hodgkin's lymphoma were enrolled and treated with an escalated dose based on the toxicity assessment observed. Dose-limiting toxicities included febrile neutropenia, rash, mucosisits and hypokalaemia. The initial phase I study reported significant QTc prolongations resulting in one grade 5 toxicity but further work has established a safe tolerated dose without this important toxicity and phase II trials are on going(Mahadevan, Plummer *et al.* 2011) (Chen, Hotte *et al.* 2014).

1.7.6 Conclusion

The role of CDKs and their inhibition in cancer including pancreatic cancer has shown therapeutic promise. AT7519 a general CDK inhibitor has shown efficacy in a range of tumours in the pre clinical setting and therefore is agent that warrants evaluation in PDAC as an agent that may offer further therapeutic promise particularly against the inherent gemcitabine resistance seen.

1.8 HSP90

Heat Shock Protein 90 (HSP90) is one of the most abundant molecular chaperones crucial in the function and survival of eukaryotic cells. HSP90 is required for the stability and function of a number of signalling proteins and other key regulators of the cell cycle. It facilitates the final activation of selected client proteins rather than *de novo* protein folding and therefore does not only function in relation to protein folding but also contributes in a host of other cellular processes including protein degradation, signal transduction and intracellular transport. There are two main genes that encode HSP90 protein (α and β), α is induced in times of cellular stress and β is mainly involved in regulating clients under normal conditions. This means that α is of particular relevance to the cancer environment where stresses such as hypoxia, nutrient deprivation and acidosis exist (Taipale, Jarosz *et al.* 2010) (Nathan, Vos *et al.* 1997).

1.8.1 Structure of HSP90

HSP90 acts as a homodimer with three flexibly linked functional regions including an N-terminal ATP-binding domain (N-domain), a middle domain (M-domain) and a C-terminal dimerization domain (C-domain) and resides primarily in the cytoplasm of eukaryotic cells; although, there are organelle specific HSP90 variants such as TRAP1 in the mitochondria and GRP94 in the endoplasmic reticulum(Sreedhar, Kalmar et al. 2004). The ATP-binding site and structural conformity is conserved from bacteria to man aside from a cross link between the N-terminal and M-domain. The M-

domain is active in ATP-hydrolysis and contains catalytic components for forming the ATPase site (Meyer, Prodromou *et al.* 2004). The C-domain is essential for dimerizastion (Ratzke, Mickler *et al.* 2010).

1.8.2 Functional conformity

HSP90 is a ATP-dependant molecular chaperone functioning in a cycle associated with ATP binding and hydrolysis. Structurally HSP90 adopts two distinct functional conformities that are in a dynamic equilibrium. In the inactive unbound state the protein is in an open V-shaped conformation. The binding of ATP triggers a number of conformational changes and HSP90 reaches its compact, closed state. The fact that these states are in dynamic equilibrium remains important functionally as this may allow HSP90 to adapt to different client proteins (Li and Buchner 2013). The protein binding region is located towards the C-terminal, it is the ATP hydrolysis that drives the conformational changes above described that govern the appearance of the protein binding site (Grenert, Sullivan *et al.* 1997). In the open confirmation HSP90 has some hydrophobic residues exposed that recruit unfolded or misfolded proteins with the same areas. Once the bound substrate is in position the ATP hydrolysis near the N-terminal results in the conformational changes that clamp down onto the substrate. This ability of HSP90 allows several functions including protein folding assistance, aggregation prevention and as a transport mechanism (Wegele, Muller *et al.* 2004). HSP90 functions as part of a multichaperone complex in association with cochaperones and client proteins. In the open state a client protein is clamped onto HSP90 with the assistance of

cochapernes, for example HSP70 and HSP40. Moving to the closed state Cdc37, p23 and immunophilins replace the original cochaperones to facilitate maturation of the conformation of the client and therefore maintain the protein in its active state in order to exert a function(Prodromou and Pearl 2003). The structure and functional conformity of HSP90 is summarised in figure 1.6.

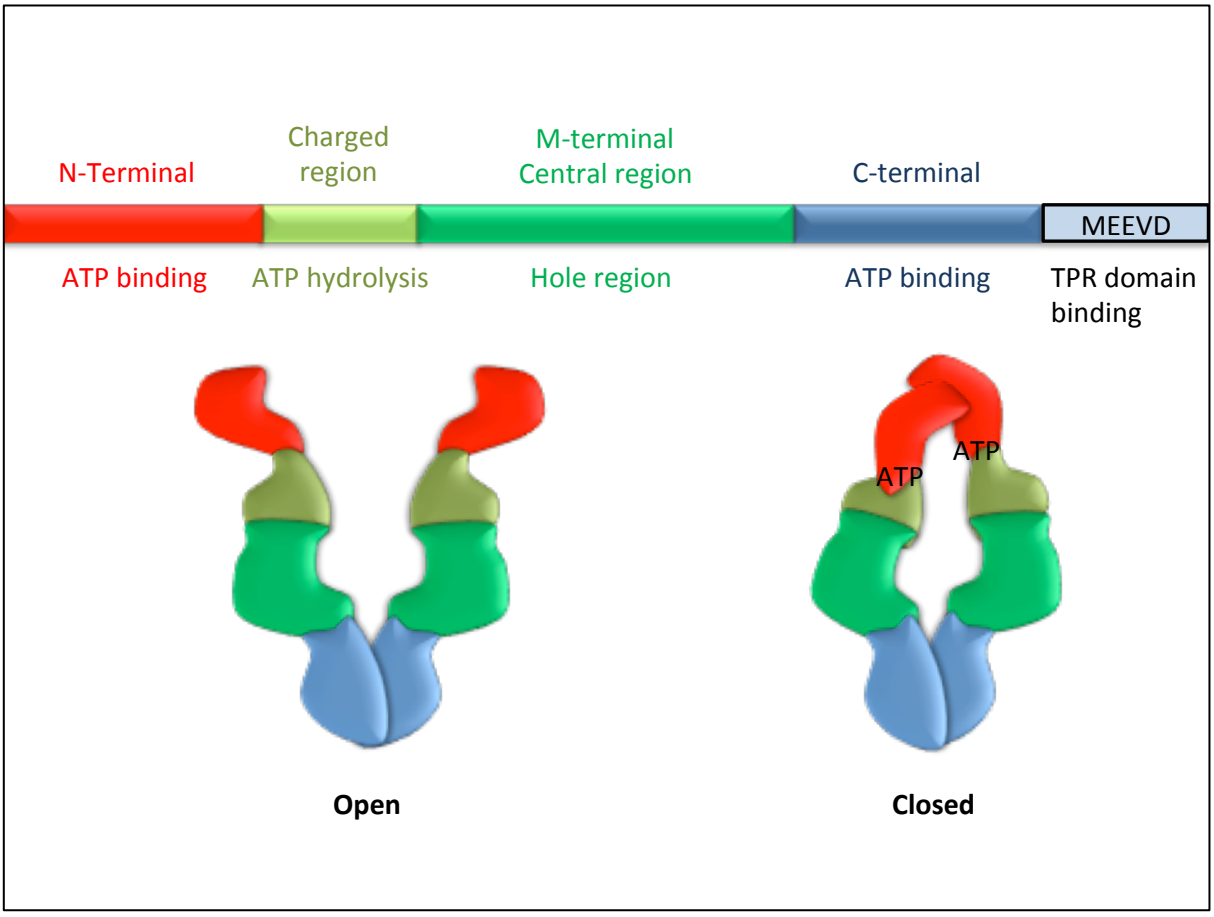


Figure 1.6 The structure and functional conformity of HSP90.

1.8.3 HSP90 in cancer biology

In cancer biology client proteins have been identified that associate with HSP90, those described include EGF receptors, MET, Raf-1 kinase, AKT, mutant p53, CDK4 and Bcr-abl(Maloney and Workman 2002; Neckers and Ivy 2003). These client proteins rely on HSP90 for stabilisations and/or maturation and contribute to the hallmarks of cancer, as described by Hanahan and Weinberg (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). The hallmarks include self-sufficiency in growth signalling, insensitivity to anti-growth signalling, evading apoptosis, sustained angiogenesis, metastasis and unlimited proliferative potential. In pancreatic cancer 12 pathways that are routinely mutated have been described, observations showed some cancer had mutations in all these pathways and the majority had mutations in at least 9 (Jones, Zhang *et al.* 2008). It follows that proteins appearing on more than one of these pathways provide interesting targets for therapy and it is noted that HSP90 is required for all of these pathways(Xu and Neckers 2007; Taldone, Gozman et al. 2008). Pancreatic cancer remains a complicated disease possessing multiple genetic changes. Over 90% of pancreatic ductal adenocarcinomas have a mutation in the *KRAS* gene that *restricts self-inactivation* (Eser, Schnieke *et al.* 2014), its protein that can affect both the PI3K/AKT and MAP kinase pathways. HSP90 client proteins AKT, and EGFR are both over-expressed, amplified or activated in 60% (AKT2) and 70% of pancreatic cancers respectively(Ghaneh, Costello *et al.* 2007). More generally both the MAP kinase pathway and the PI3K/AKT pathways have been associated in pancreatic cancer (Jones, Zhang *et al.* 2008) (Reichert, Saur *et al.* 2007). Additionally pancreatic

cancer, in common with many cancer, have increased levels of HSP90 and may rely more heavily on the HSP90 protein to stabilize the mutated and activated proteins on which they depend. HSP90 has been found to be expressed 6- to 7-fold higher in human pancreatic cancer than in normal tissue (Ogata, Naito *et al.* 2000). Thus the targeting of multiple pathways through the inhibition of HSP90 is a more attractive target than that of a single molecule in the treatment of such a complex disease as pancreatic cancer. Table 1.10 summarises the HSP90 client proteins associated with oncogenesis.

HSP90 client proteins associated with oncogenesis	
Kinases	Akt/PKB Bcr-Abl CDK4, CDK6, CDK9 Death domain kinase RIP ErbB2 (and mutant EGF receptor) C-MET IGFR MEK MOK, MAK, MRK PDK 1 Pim-1 Plk 1 FAK Pp60v-src, c-src Raf-1, B-Raf, Ste 11 trkB VEGFR2 Weel, Swel
Transcription factors	Steroid receptors (GR, MR, ER, PR, AR) HSF-1 p53 Stat3
Others	Apaf-1 Mdm2 Proteasome Ral-binding protein 1 Survivin SV40 large T antigen Telomerase

Table 1.10 A table showing HSP90 client proteins associated with oncogenesis, adapted from (Zhang and Burrows 2004).

1.8.4 Inhibition of HSP90

The desirable feature of HSP90 as a target for cancer therapy stem from its position on multiple cancer related pathways(Workman 2004). The fact that its actions are multifactorial (figure 1.7) should also significantly reduce the opportunity for cancer cells to become resistant to HSP90 inhibition.

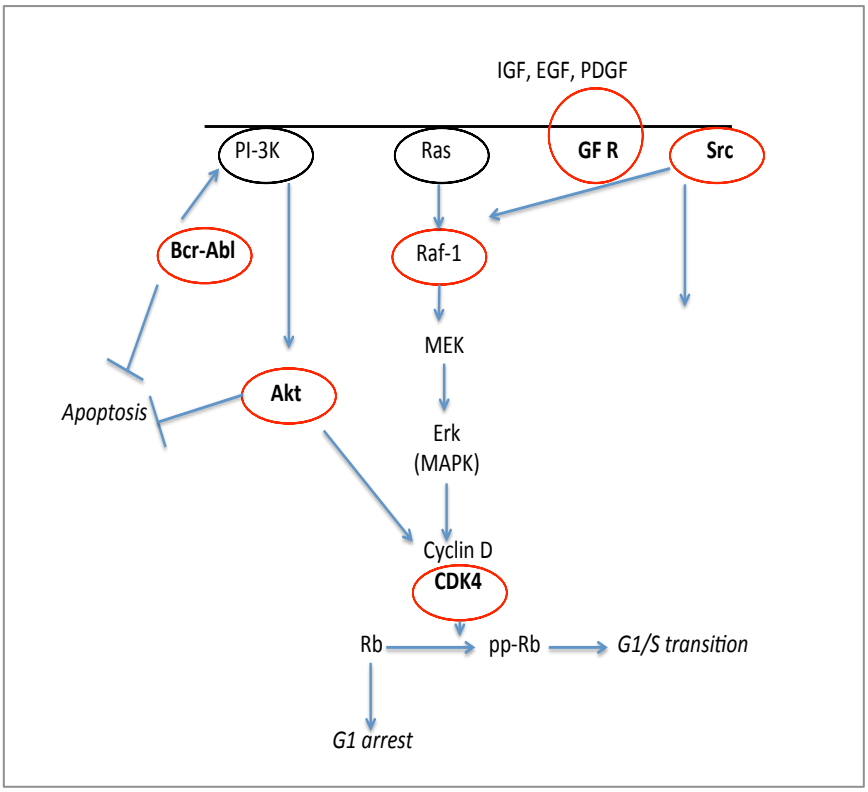


Figure 1.7 The multiple signalling pathways disrupted by HSP90 inhibition disrupting both anti-apoptotic signalling and cell cycle progression. Targets of HSP90 inhibitors are circled. Adapted from Blagosklonny et al.(Blagosklonny 2002).

It is obviously a concern that HSP90 is pivotal not only cancer but also normal cell function: i.e. toxicity may be an issue. The therapeutic selectivity of HSP90 inhibition in cancer relies on a number of factors (Workman, Burrows *et al.* 2007). Firstly the fact that cancer cells become reliant on the oncogenic pathways that drive the malignancy, in turn depleting oncoproteins has a much greater effect on cancer cells than normal tissue (Weinstein and Joe 2006). Secondly, oncoproteins in cancer often exist in their mutated form and are much more reliant on HSP90 than in their normal state (Grbovic, Basso *et al.* 2006). The cancer microenvironment

(including acidosis and hypoxia) is heavily influenced by molecular chaperones, in such an environment HSP90 is often involved in large muticomplexes as opposed to the uncomplexed HSP90 found in healthy cells. The inhibitors developed can specifically target the bound form of HSP90, such as 17-AAG (Kamal, Thao *et al.* 2003).

Geldanamycin and its derivatives 17-allyamino-geldamycin (17AAG) and 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (DMAG) are HSP90 inhibitors that initiated interest in the inhibition of HSP90 as a strategy for cancer treatment. Geldanamycin is a benzoquinone asamycin that has been shown to bind directly with HSP90 interfering with the heterocomplex formation and ATP binding to the N-terminal resulting in ubiquitin mediated proteasomal degradation of its client proteins(Workman, Burrows *et al.* 2007) (Whitesell, Mimnaugh *et al.* 1994) (Grenert, Sullivan *et al.* 1997). Despite the potent anti-tumour effects demonstrated by Geldamycin and 17AAG their use was limited by hepatotoxicity and water solubility (Usmani2009) (Banerji, O'Donnell *et al.* 2005). 17-AAG has been investigated in prostate cancer and melanoma in a Phase II study but no objective clinical responses were seen with the dose and scheduling deemed safe in phase I studies (Heath, Hillman *et al.* 2008) (Solit, Osman *et al.* 2008). 17-DMAG is a geldanamycin derivative with substitution of the C-17 methoxy group that resulted in increased water solubility and bioavailability (Hollingshead, Alley *et al.* 2005). In phase I trials clinical responses or stable disease have been observed in prostate, melanomas and renal cell carcinomas but again drug-related toxicities

were observed limiting its role moving forward (Pacey, Wilson *et al.* 2011) (Kummar, Gutierrez *et al.* 2010).

Another mechanism for HSP90 inhibition is disruption of the bond between HSP90 and its co-chaperone Cdc37. This was investigated by Zhang *et al* using celastrol, they demonstrated inhibition of cell division and induction of apoptosis in pancreatic cancer cells lines. In an *in vivo* model celastrol inhibited pancreatic tumour growth by up to 80% ($p < 0.001$) (Zhang, Hamza *et al.* 2008).

Structure based approaches have also been employed in the development of HSP90 inhibitors. Based on the purine scaffold PU3 was designed and reported on as the first synthetic HSP90 inhibitor (Chiosis, Timaul *et al.* 2001). Since its development a number of other agents have been developed based on this initial prototype molecule and advanced to clinical trials. CNF2024/BIIB021 as one such agent has been investigated in phase I and II clinical trials in advanced solid organ malignancies, toxicities included grade 3 and 4 fatigue, hyponatreamia and hypoglycaemia and efficacy investigation demonstrated stable disease in 11 of the 16 patients studied (Saif, Takimoto *et al.* 2014).

Another HSP90 inhibitor that has been investigated is novobiocin although this inhibition was an additional effect not originally expected. Novobiocin is a aminocoumarin antibiotic that acts through the inhibition of DNA gyrase and was originally investigated as an agent for the treatment of bacterial infections(Samuels

and Garon 1993). It has also been shown to provide a degree of HSP90 inhibition by binding to the HSP90 C-terminal binding site and subsequently induce degradation of client proteins(Donnelly and Blagg 2008). The activity of novobiocin itself in relation to HSP90 inhibition was weak therefore a number of analogues were developed that have shown more promising anti-cancer activity (Burlison, Neckers et al. 2006). Montori et al demonstrated one such analogues activity in breast cancer cell lines(Montoir, Barille-Nion et al. 2016) as well as other literature demonstrating novobiocin analogues activity in lung and breast cancer cell lines(Hall, Seedarala et al. 2016)

The investigation of HSP90 inhibitors and issues experienced have resulted in the development of compounds that are less toxic more water-soluble and have better pharmacodynamics including AT13387 (woodhead2010).

1.8.5 AT13387- A novel HSP90 inhibitor

AT13387 is a small-molecule inhibitor of HSP90 developed by Astex Pharmaceuticals Inc. via fragment based drug discovery (figure 1.8). Fragment based drug discovery is a process based on identifying small chemical fragments that bind to a biological target then combining or growing them to produce a lead compound with a high affinity for the proposed target. The resulting compounds have benefits of higher ligand efficiency with low molecular weight compounds (Murray, Carr *et al.* 2010). Fragment screening using NMR and high throughput X-ray crystallography was used

to develop AT13387 as a novel inhibitor of HSP90. It was demonstrated to have a high affinity for HSP90 binding at the ATPase site of the N-terminal which was demonstrated to have inhibitive activity against a range of human cancer cell lines (table 1.11) and cytostatic activity in a xenograft model (Woodhead, Angove *et al.* 2010).

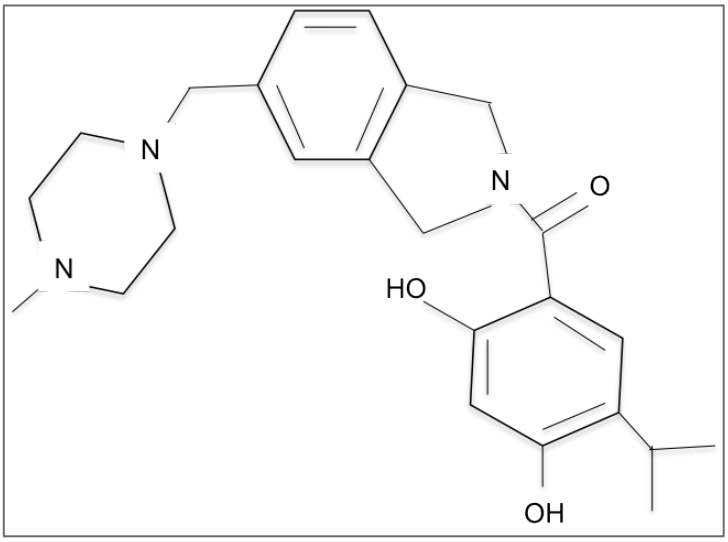


Figure 1.8 The structure of an AT13387 molecule adapted from Woodhead et al (Woodhead, Angove et al. 2010)

There has already been published data on its efficacy in a range of cancers from *in vitro* and *in vivo* models. Smyth *et al* investigated AT13387 activity against an *in vitro* and *in vivo* gastrointestinal stromal tumour model and found it effective both in Imatinib (standard therapy) sensitive and resistant cell lines, notably AT13387 was effective in cell lines resistant to 17-AAG, an alternative HSP90 inhibitor (Smyth, Van Looy *et al.* 2012). As a result of these initial investigations a randomised phase II study of patients with advanced GIST tumours who have progressed following

standard therapy has been completed using AT13387 both as a single agent and in combination with Imatinib, the results are yet to be published.

Graham *et al* demonstrated AT13387 efficacy in a xenograft model of refractory non-small cell lung cancers demonstrating significant reduction in tumour growth compared to controls and noted a long duration of action both *in vitro* and *in vivo* (Graham, Curry *et al.* 2012). Currently phase I and II clinical trials have been completed in Gastrointestinal Stromal Tumours (GIST) (Mahadevan 2013)and a phase I trial completed in refractory solid organ tumours demonstrating tolerability only grade 2 adverse events (Mahadevan 2012).

More recently AT13387 has been demonstrated to overcome issues in melanoma relating to clinical resistance to BRAF inhibitors in pre-clinical studies (Smyth, Paraiso *et al.* 2014) and further drug safety in a phase I clinical trial of patient with advanced solid organ tumours has demonstrated safety and tolerability with preliminary anti-tumour activity (Shapiro, Kwak *et al.* 2014). Current phase II studies continue in the treatment of non-small cell lung cancer and BRAF melanoma.

Origin	Cell Line	AT13387 GI50 (nM)
Normal Prostate	PNT2	480
Colon Carcinoma	HCT116	48
	HT-29	78
	SW620	210
Lung Carcinoma	A549	22
	NCI-H1975	27
	NCI-H1993	63
	NCI-H1650	13
Breast Carcinoma	MCF-7	53
	MDA-MB-231	260
	MDA-MB-468	25
	SK-BR3	63
	T47D	29
	BT474	13
Multiple Myeloma	U266	58
	RPMI 8226	70
Hepatoma	Huh-7	22
Prostate Carcinoma	DU145	94
	PC3	120
	LNCaP	77
	22Rv1	46
Uterine Sarcoma	MES-SA	53
	MES-SA/Dx5	42
Ovarian	SKOV3	44
Leukaemia	HL60	22
	K562	47
	MV4-11	13
Melanoma	A375	18
	SkMel 28	44
Cholangiocarcinoma	TFK-1	19

Table 1.11 A table showing the anti-proliferative effect of AT13387 on a panel of human tumour cell lines with a notable absence of any description of PDAC lines, adapted from Graham et al (Graham, Curry et al. 2012)

1.8.6 Conclusion

Targeting HSP90 inhibition offers a promising therapeutic avenue in PDAC owing to the complex molecular events seen in this disease. AT13387 as a novel HSP90 inhibitor presents a new agent that warrants investigation in PDAC in the pre-clinical setting, it offers new treatment that may be able to enhance the gains of current therapy and overcome the innate gemcitabine resistant seen in this disease.

1.9 STUDY DESIGN AND OBJECTIVES

1.9.1 Aims

The aim of this study was to evaluate two novel agents in pancreatic cancer, AT7519, a general CDK inhibitor, and AT13387, a HSP90 inhibitor as single agents and in combination with standard therapy (gemcitabine). The actions of each agent alone and in combination with standard chemotherapy (gemcitabine) *in vitro* was investigated in pancreatic cancer cell lines (including a gemcitabine resistant cell line), and their efficacy *in vivo* was evaluated.

1.9.2 Study design *in vitro*

1. Standard pancreatic cancer cell lines were cultured as well as a gemcitabine resistant line for *in vitro* experimentation.
2. The IC50 of AT7519, AT13387 and gemcitabine was determined on a range of pancreatic cancer cell lines using MTS assay.
3. The effects on the cell cycle by each agent (and AT7519/AT13387 in combination with standard therapy) was investigated using flow cytometry.
4. Evidence of induction of apoptosis of AT7519 and AT13387 was investigated using the caspase 3/7 assay.
5. Isobolar analysis of AT7519 + gemcitabine and AT13387 + gemcitabine was undertaken using MTS assay.

6. Western blotting was undertaken to measure biomarkers related to assumed drug actions for both single agents (AT7519 or AT13387) and agents in combination with standard therapy (gemcitabine)

1.9.3 Study design *in vivo*

1. Subcutaneous xenografts of Suit2 and Miapaca-2 cell lines were established in nude mice and the cell line that produced tumours most amenable to a xenograft model was taken forward to further studies.

2. AT7519, AT13387 and gemcitabine were assessed for tolerability as single agents.

3. Responses to AT13387, AT7519 and gemcitabine were assessed as single agents against control animals measured by tumour volume.

4. AT7519 + gemcitabine and AT13387 + gemcitabine were assessed for tolerability in combination.

5. Responses to AT7519 and AT13387 in combination with gemcitabine were assessed for efficacy against control animals using tumour volume as the primary endpoint.

CHAPTER 2: MATERIALS AND METHODS *IN VITRO*

2.1 DRUG TREATMENTS

2.1.1 Gemcitabine

Gemcitabine for *in vitro* experiments was derived from a master stock already available in the department (diluted by K Dajani). Powered gemcitabine had been purchased from Eli lilly Ltd (Basingstoke, UK) and a stock solution of 0.1M was made with Dulbecco's Phosphate Buffered Saline (PBS) (Sigma, Poole, UK). This was filter sterilized and stored at -80ºc in 0.5ml aliquots.

2.1.2 AT7519

AT7519 was provided by Astex Pharmaceuticals (Cambridge, UK). Powered agent was diluted in Dimethyl sulfoxide (DMSO) (Sigma, Poole, UK) to a stock solution concentration of 10 mM and kept in 20-50µl aliquots at room temperature for a maximum of 6 weeks (as recommended by Astex Pharmaceuticals).

2.1.3 AT13387

AT13387 was provided by Astex Pharmaceuticals(Cambridge, UK) . Powered agent was diluted in DMSO into a stock solution of 10 mM and kept in 20-50µl aliquots at room temperature for a maximum of 6 weeks (as recommended by Astex Pharmaceuticals).

2.1.4 Dilution of stock solutions

When required for experimentation further dilutions of the master stock solutions were performed with Roswell Park Memorial Institute medium (RPMI) (Sigma, Poole, UK) tissue culture media for all agents.

2.2 *IN VITRO* CELL STUDY

2.2.1 Standard cell lines

The pancreatic cancer cell lines used, their origin and source for this study are detailed in table 2.1.

Cell line	Origin	Sourced from
SUIT-2	metastatic liver tumour of human PDAC	Stores in department of molecular and clinical cancer medicine
Miapaca-2	PDAC obtained from a 65-year-old Caucasian male	Stores in department of molecular and clinical cancer medicine
Panc-1	PDAC from a 56-year-old Caucasian male	ATCC via LGC standards (Middlesex, UK)
CFPac	metastatic liver lesion of PDAC in a 26 year old Caucasian male with cystic fibrosis (CF)	Stores in department of molecular and clinical cancer medicine
Fampac	poorly differentiated PDAC in a 43-year-old Caucasian female with a familial predisposition to pancreatic carcinoma	Stores in department of molecular and clinical cancer medicine

Table 2.1 A table of the pancreatic cancer cell lines used in experiments with their original origin and the supplier used.

These pancreatic cancer cell lines have been characterised and information on their genetic alterations are detailed in table 2.2.

Cell line	KRAS alteration and product	p53 alteration and product	p16 alteration and product	Tumour marker Expression
SUIT-2	Mutant ASP 12	Mutant His 273	69 GAG-TAG Glu-stop	Ca19-9 CEA
Miapaca-2	Mutant 12 cys	Mutant 248 trp	Homozygous deletion No product	
Panc-1	Mutant Asp 12	Mutant His 273	Homozygous deletion No product	
CFPac	Mutant Val 12	Mutant Arg 242	Methylated No product	Ca19-9 CEA
Fampac	Mutant Gly > Val 12	Mutant	Homozygous deletion No product	

Table 2.2 A table demonstrating the pancreatic cancer cell lines genetic mutations and tumour marker expression.

2.2.2 Gemcitabine resistant cell line

A gemcitabine resistant cell line, SUIT-2 (GR), was established in the department by Khaled Dajani (Department of Molecular and Clinical cancer medicine) and a subsequent clonal population developed by Elisabeth Shaw (Cancer Research

Centre UK, Liverpool). SUIT-2 cells were rendered gemcitabine resistant by sub-culturing with progressively increasing concentrations of gemcitabine to a plateau of 5 mM for maintenance. This provided a mixed population of adaptive and clonally resistant cells.

The clonal population was separated by growing small numbers of cells in 96 well plates and on going culture of those that maintained a large cell population in 5µM of gemcitabine. These cells were tested for on going gemcitabine resistance by sub-culturing in the absence of gemcitabine and MTS assays performed to establish the IC50 value.

2.3 TISSUE CULTURE

All tissue culture work was undertaken in a class II hood with laminar flow which was cleaned with 70% ethanol prior to use. Tissue culture reagents (RPMI media, PBS and Trypsin, all from Sigma, Poole, UK) were warmed in a 37^{o}C water bath for 15 minutes prior to use and bottles cleaned with 70% ethanol prior to transfer to the hood. All tissue culture work was performed using a no touch technique and asepsis ensured as feasible.

2.3.1 Defrosting cells

Vials of the required cell line pellet were retrieved from either -80^{o}C stores or liquid nitrogen stores. The vial of cell pellet was rapidly defrosted and spun in a centrifuge

at 500 revolutions per minute (rpm) for 5 minutes to remove DMSO. The resultant cell pellet was then diluted into standard RPMI media to 10ml and transferred to a T25 flask(Sigma, Poole, UK). Once cells were growing adequately they were transferred to T75(Sigma, Poole, UK) flasks on subsequent splits.

2.3.2 Sub-culturing cells

Cells contained in a T25 or T75 flask had RPMI media removed and the adherent cells were lavaged with 10ml of warmed PBS, which was subsequently discarded into virkon. Trypsin was then introduced into the flask, 1ml per T25 and 2ml per T75, and the flask returned to the incubator for the time required to lift the adherent cells from the surface. After this period of time flasks were removed from the incubator back to the hood and trypsin diluted with RPMI media to make a total volume of 10ml (i.e. 9ml of media per T25 and 8ml of media per T75). Cells were then dispensed into new flasks at the desired concentration. Fresh RPMI media was then added to make a total volume of 10ml per T25 and 20ml per T75. The routine practices for each cell line used are detailed in table 2.3.

Cell line	Time for trypsining cells (minutes)	Routine sub-culture split
SUIT-2	3	1 in 10
Miapaca-2	3	1 in 10
CFPAC-1	5	1 in 5
Panc-1	1	1 in 5
Fampac	5	1 in 5
SUIT-2 (GR)	3	1 in 10

Table 2.3 A table showing the pancreatic cancer cell lines average times for trypsinising and the sub-culture spilt routinely used for cell line maintenance.

2.3.3 Freezing cell stocks

Adherent cells in a T75 flask at approximately 80% confluence were used to make frozen cell stocks. Cells were firstly lavaged with 10ml of PBS that was subsequently discarded into virkon. 2ml of trypsin was added to each flask and the flask returned to the incubator for the desired time to lift adherent cells. After this time the cell flask was returned to the hood and trypsin diluted in 8ml of media to make a total volume of 10ml. The 10ml cell solution was then transferred into a 15ml eppendof (Sigma, Poole, UK) and spun in a centrifuge at 500rpm for 5 minutes. Next the

media was removed from the resultant cell pellet and then this was resuspended in 10ml of PBS and spun for a further 5 minutes at 500rpm. The PBS was then removed and the cell pellet mixed with 5-10ml of freezing media (dependent on the size of cell pellet obtained at this stage). The cell solution was then transferred to labelled cryotubes in 1ml aliquots. Cryotubes (Sigma, Poole, UK) were left on ice for 1 hour then transferred to -80^{o}C for 48 hours and subsequently liquid nitrogen stores (if for long term storage).

2.3.4 Authentication and Mycoplasma screening

All cells lines used were verified as the correct line by genotyping via PCR (powerplex16, promega, Southampton, UK) by E. Shaw. Cells were regularly (approximately 3 monthly) screened for mycoplasma using PCR by E. Shaw.

2.4 MTS ASSAY

In order to determine the optimal seeding density of cells on Corning 96 well plates (Sigma, Poole, UK) as well as the IC50 of the drug treatment and effects of drugs in combination the EZ4U (Biomedica, Oxford, UK) assay was used. EZ4U is a non-radioactive cell proliferation and cytotoxicity assay that measures the cells ability to convert the yellow coloured tetrazolium compound into its red formazan derivative. This is an active process that requires functioning mitochondria therefore the assay is a measure of cell vitality and an indirect measure of cell death. The kit consists a SUB Substrate power, 1 vial of which is enough solution for 2 96 well plates with 100µl per well and ACT activator solution. 2.5ml of activator solution that was pre-

warmed to 37^{o}C was added to one vial of SUB substrate power, which dissolved to yield a straw coloured solution and was used immediately. 10µl of the dye solution was added to each 100µl well and absorbance readings were taken from 0 to 4 hours at hourly intervals with incubation at 37^{o}C between readings. These were taken on a microtiterplate reader set at 450nm with 620nm as a reference used to correct any non-specific background values. The plates were shaken by the plate reader prior to each reading in a standard fashion.

The numerical values produced for each well were averaged across wells with the same conditions and used to give a difference compared to the control wells and corrected against blank wells (i.e. those containing no cells).

2.4.1 IC50 determination

Determination of optimum seeding density (the density to reach approximately 80% confluence in 24 hours) to commence experiments for each cell line was first determined by seeding cells at variable densities on a 96 well plate then performing MTS assays at 24, 48, 36, 72 and 96 hours. Once the optimum seeding density was established for each cell line cytotoxicity assays were performed. Cells were seeded at the required density in 100µl in 96 well plates and incubated for 24 hours. Subsequently the media was removed a fresh media with varying concentrations of drug were added and plates then incubated for 24, 48, 36, 72 and 96 hours at which points MTS assays were performed A typical example of a plate for these

experiments is shown in figure 2.1. IC50's for each cell line were done in triplicate. The IC50 for each experiment was determined by plotting the average growth curve for well across each plate compared to the percentage controls and the 50% point of cell growth was determined and the IC50 derived.

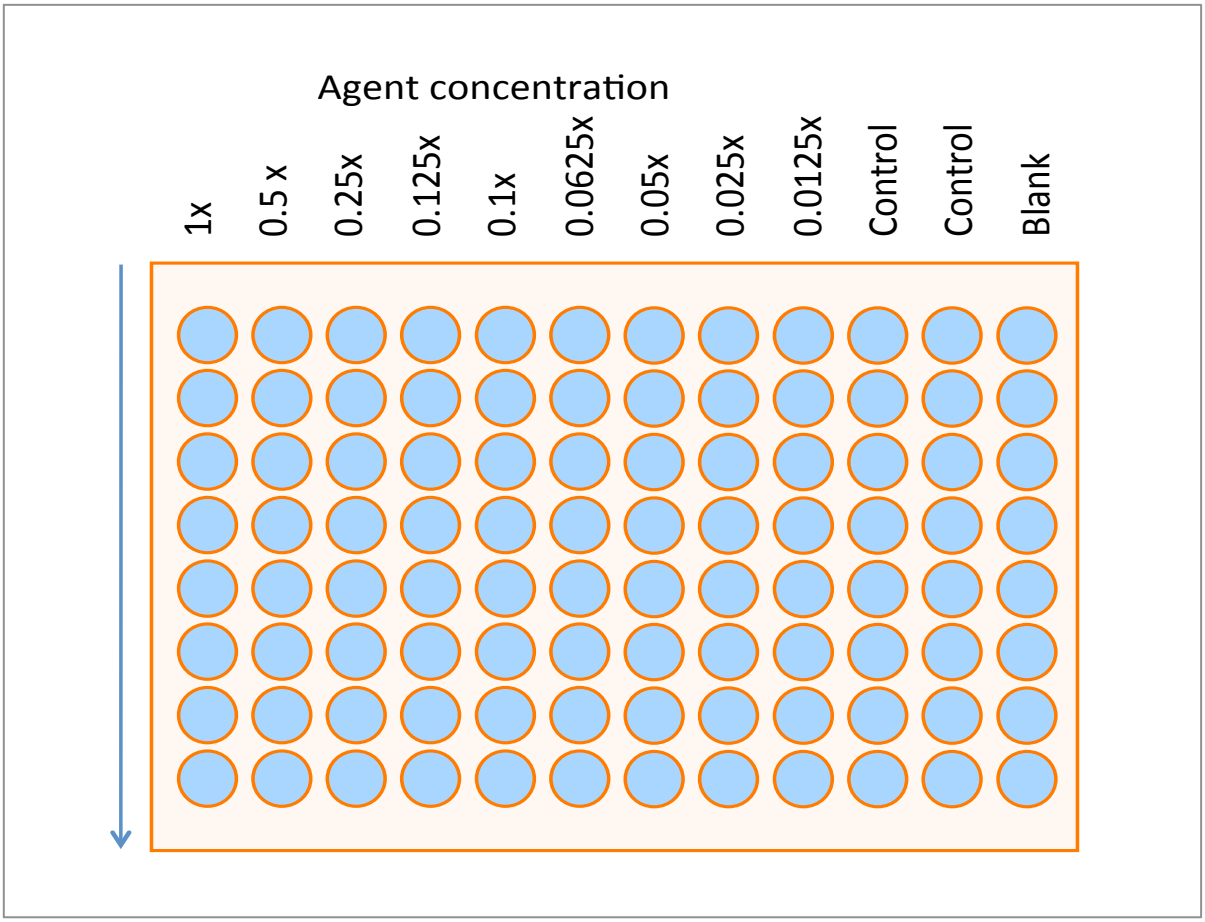


Figure 2.1 A figure showing a 96-well plate configuration to determine the cytotoxicity of agents against pancreatic cancer cell lines.

2.4.2 Isobolar analysis of drug combinations

Isobolar analysis of drug combination were completed for Gemcitabine + AT7519 and Gemcitabine + AT13387. The concentrations of agent used were determined from the single agent IC50's and the IC50 was used as the 0.25x or 0.5x concentration on the isobolar plates (i.e. the 1x concentration was 4x or 2x the

IC50). Cells were seeded in 96 well plates at previously determined optimum seeding densities then 24 hours later media removed and drug media introduced. Variable dosing schedules in combination were used as in table 2.4 and assays were completed with a 48 and 72 hour MTS reading.

	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**
Design 1	seeding cells	agent 1 +agent 2 introduced		MTS assay (48 hours)	
Design 2	seeding cells	agent 1 introduced	agent 1 +agent 2 introduced		MTS assay (48 hours)
Design 3	seeding cells	agent 2 introduced	agent 1 +agent 2 introduced		MTS assay (48 hours)

Table 2.4 The timeline for 3 experimental designs of isobolar analysis.

Isobolar plates were prepared in a standard format, an example of which is shown in figure 2.2.

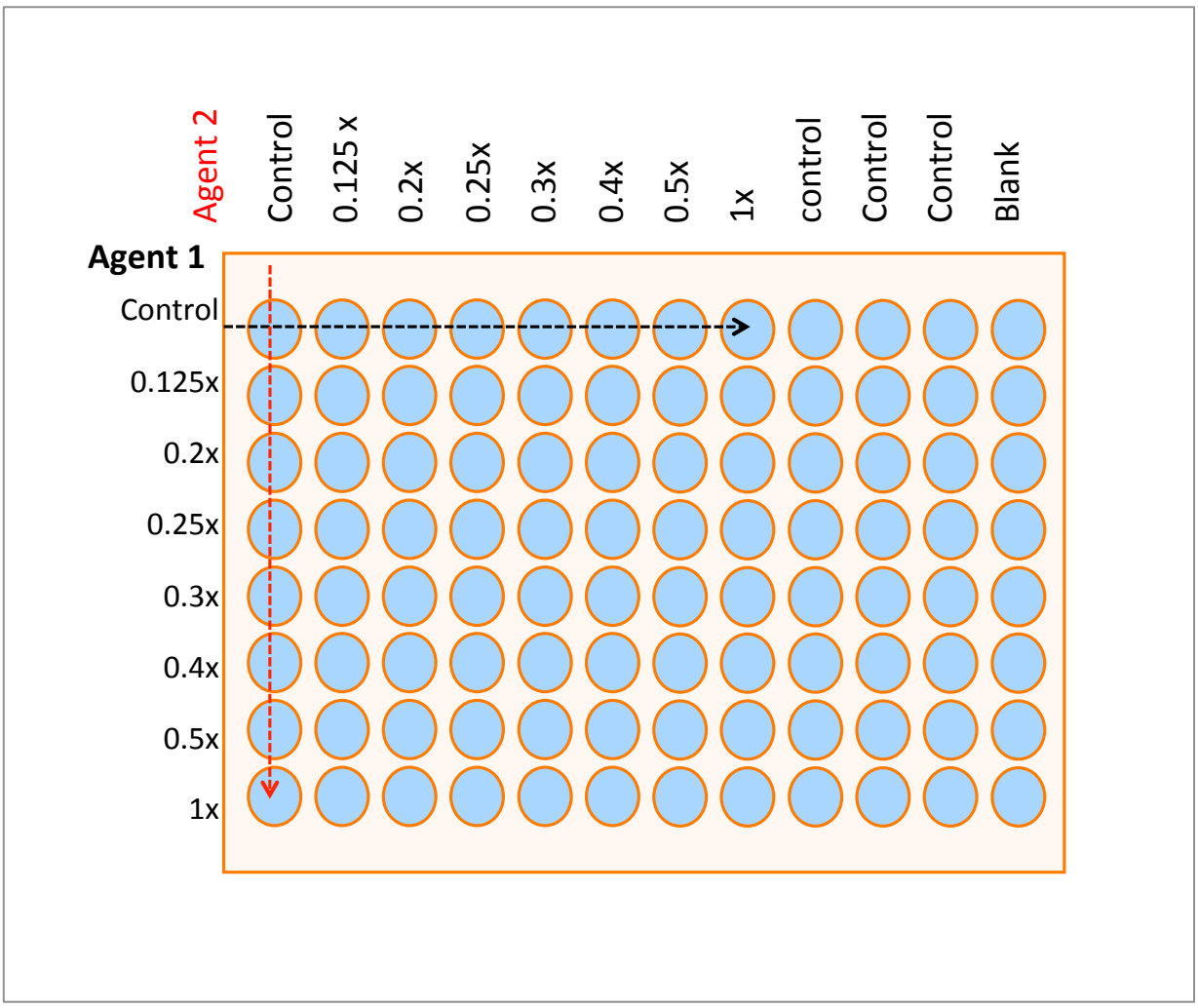


Figure 2.2 The 96-well plate configuration for isobolar analysis.

2.5 CELL CYCLE ASSAYS

Cell cycle analysis was completed on pancreatic cancer cell lines using untreated cells and drug treated cells performed using propidium iodide (PI) (Sigma, Poole, UK) staining and flow cytometry.

2.5.1 Cell preparation

Suit2 cells were seeded onto 6 well plates at 2.5 x 10^5 cells per well in RPMI media and incubated for 24 hours.

2.5.2 Drug treatment

After 24 hours incubation RPMI media was removed and cell treated with AT7519, AT13387, gemcitabine, a combination of Gemcitabine + AT13387, Gemcitabine + AT7519 or control (0.1% DMSO or PBS). Experiments were completed with varying drug doses and times of treatment.

2.5.3 Cell preparation for PI staining

Both adherent and floating cells were collected and washed with twice with PBS prior to fixation in 70% ethanol overnight before staining with PI (50ug/ml) for 15minutes at room temperature.

2.5.4 Assay measurement

Propidium iodide incorporation was using assessed using a CyAn™ ADP Analyzer (Beckman Coulter, High Wycombe, UK). Duplicates were formed each time and experiments were repeated in duplicate on different days. The resultant data was statically analysed using statview calculating mann-whitney generated p values.

2.6 APOPTOSIS ASSAYS

To determine the timing and mechanism of cell death caspase 3/7 activity was assessed using the Caspase-Glo 3/7 luminescent assay Promega, Southampton, UK).

2.6.1 Cell preparation

Miapaca-2 pancreatic cancer cell line were seeded in 96 well plates at 5000 cells per well and incubated for 24 hours to adhere to the plate in RPMI media.

2.6.2 Treatment of cells

RPMI media was removed and fresh media with either 10μM AT7519, 1μM AT13387 or 0.1% DMSO control was introduced and cells were incubated for a further 6, 16, 24, 40, 48 or 72 hours.

2.6.3 Caspase 3 assay

Caspase-Glo reagent was added, as detailed in the manufacturers protocol using 100ul volumes and incubated for 1 hour. Luminescence was then measured in a microplate reader and normalised to untreated control. A single experiment was performed with 6 replicates for each time point. Differences between control and treated cells were examined statistically using mann-whitney p values via statview.

2.7 WESTERN BLOTTING

Western blot analysis was completed on pancreatic cancer cell line lysates. Western blots were completed on untreated cells and drug treated cells with variable drug concentrations, sampled at variable time points.

2.7.1 Antibodies

The antibodies used as potential downstream markers in western blotting were supplied by Cell Signalling Technology via New England Biolabs ltd, Hertfordshire, UK.

The antibodies used for AT7519 were cdc2, pp1-α, phospho-pp1-α, Rb, phospho-Rb, NPM and phospho-NPM (table 2.5). Cdc2 antibody detects endogenous levels of cdc2 protein, which encodes for CDK1. Pp1-α and its phosphorylated version are involved in cell cycle regulation. Pp1-α dephosphorylates Rb and cdc25 during mitosis and its cell-cycle dependant phosphorylation by cdc2 kinase inhibits its activity. Retinoblastoma tumour suppressor protein (Rb) regulates proliferation by controlling the restriction point within the G1 phase of the cell cycle and cell cycle dependant phosphorylation by CDKs inhibits its activity, therefore allowing cell cycle progression. Nucleophosmin (NPM) is a phosphor protein abundant primarily in the nucleus and an elevated level is often found in tumour cells. NPM had multiple functions including centresome duplication as a substrate of CDK2/cyclinE, its phosphorylation is a prerequisite step for centrosome to initiate duplication. The Antibodies used for AT13387 were HSP70, S6, phospho-S6, CDK 4, Akt, phospho-Akt and Raf-1 (table 2.6).

Target	Size	Antibody code	species	Dilution	Antibody incubated in (TBS)	Blocking agent (TBS)
Cdc2	34kDa	9112	Rabbit	1 in 2000	5% BSA	5% BSA
Pp1α	38kDa	2582	Rabbit	1 in 1000	5% BSA	5% BSA
phospho-pp1α	38kDa	2581S	Rabbit	1 in 1000	5% BSA	5% BSA
Rb	110kDa	9309	Mouse	1 in 1000	5% Milk	5% Milk
Phospho-Rb	120kDa	44-574G	Rabbit	1 in 1000	5% BSA	5% BSA
NPM	38kDa	3542	Rabbit	1 in 2000	5% Milk	5% Milk
Phospho-NPM	38kDa	3541	Rabbit	1 in 1000	5% BSA	5% BSA

Table 2.5 A table showing the primary antibodies used for AT7519 experiments.

Target	Size	Antibody code	species	dilution	Antibody incubated in (TBS)	Blocking in (TBS)
HSP70*	70kDa	SPA-811	Rabbit	1 in 1000	5% BSA	5% BSA
S6	32kDa	2317	Rabbit	1 in 1000	5% Milk	5% Milk
Phospho-S6	32kDa	4838	Rabbit	1 in 1000	5% Milk	5% Milk
CDK 4	30kDa	2906	Mouse	1 in 1000	5% Milk	5% Milk
Akt	60kDa	9272	Rabbit	1 in 1000	5% BSA	5% BSA
Phospho-Akt	60kDa	4058	Rabbit	1 in 1000	5% BSA	5% BSA
Raf-1**	80kDa	Sc-133	Rabbit	1 in 1000	5% BSA	5% BSA

*HSP70 supplied by Enzo Life Sciences Ltd, Exeter, UK,
**Raf-1 supplied by Santa Cruz Biotechnology Inc, Heidelberg, Germany.
Table 2.6 A table showing the primary antibodies used for AT13387 experiments.

2.7.2 Preparation of cell lysate

Cells for western blotting were incubated for the appropriate time and conditions to the cell line in T75 flasks. Subsequently the media was removed and adherent cells washed with 10ml of PBS then trypsinised with 2mls of trypsin for the appropriate period of time at 37^{o}C. The trypsin was then neutralised with 8ml of media and the

cells in media transferred into a falcon tube and spun for 2 minutes ay 1000rpm. The media was removed from the cell pellet and this was resuspended in PBS and transferred to a 1.5ml eppindorf tube. This was centrifuged at 1000rpm for 2 minutes, the PBS was then removed and the cell pellet resuspended in 50-100µl of SDS lysis buffer dependant on the size of the cell pellet obtained. The cell lysis was then sonicaded at 13 amps for 10 seconds. If the cell lysis was not used immediately it was stored at -20^{o}C.

2.7.3 Determining protein concentration

Protein concentration of the cell lysates produced was measured using the Bradford assay. The Bradford assay is a colorimetric protein assay which is based on the absorbance of the dye coomassie brilliant blue G-250, the amount of complex present is indicated by the depth of blue colour of the solution and is a measure of protein concentration that can be quantitated by an absorbance reading.

Initially to standardise readings controls were made up of solutions with known concentrations of Bovine Serum Albumin (BSA). A stock of BSA was made up using 0.005g of bovine albumin diluted into 1ml of dH2O. The dilutions detailed in table 2.7 were then performed.

Concentration	Volume of stock BSA solution (µl)	Volume of dH2O (µl)
10	100	0
7	70	30
5	50	50
2	20	80
1	10	90

Table 2.7 A table of the dilutions of BSA used for the Bradford assay.

These standards and the cell lysates to be analysed were then placed in to eppendofs with 2µl of the sample, 200µl of Bradford assay and 798µl of dH_2O. A blank with 800µl of dH_2O and 200µl of Bradford assay was also made and was measured first to calibrate the machine then the standards and samples were measured using a spectrophotometer set at 562nm. The BSA standards were then plotted in a standard curve and a R^2 value of greater than 0.7 indicated a valid result. A formula was then applied to the samples to give the protein concentration per µl and subsequently the volume of lysate needed to load 20µg of protein per lane.

2.7.4 Preparation of protein for loading

Once the protein concentration of samples had been determined the samples were prepared ready to be loaded into each lane. This was performed either on the day of western or the day before and stored at -20^{o}C overnight. The determined volume of cell lysate was added to 3µl of loading buffer (sample loading buffer ingredients given below in table 2.8) and a variable volume of 1xRBS (dependent on the volume of cell lysate used) to make a total volume of 15µl in a 100µl eppendof. Each sample was then centrifuged to 13.3g and warmed at 96^{o}C for 15 minutes, followed by a further run in the centrifuge to 13.3g.

Sample loading buffer (1x concentration)
2% SDS
10% glycerol
60mM of Tris-HCL (pH 6.8)
0.001% bromophenol blue
200mM DDT

Table 2.8 A table showing the 1x final concentration of sample loading buffer.

2.7.5 Gels

Tris-glycine based Sodium Dodecyl Sulfate (SDS) polyacrylamide gels were cast between biorad 0.75mm plates using approximately 5ml of resolving gel and 1ml of

stacking gel per gel produced. A variable % of resolving gel was used dependant on the size of protein band expected (table 2.9).

% resolving gel	Molecular weight of band (kDa)
8	25-200
10	15-100
12	10-70

Table 2.9 A table showing the percentage gels used for differing molecular weight bands.

Initially plates were cleaned with ethanol and water then air dried. The resolving gel was pipette between the plates leaving approximately 1 inch at the top of the plates. 200µl of Isopropanol was then pipetted on top of the resolving gel to prevent bubbles and an uneven surface and the gel left to set for approximately 20 minutes. Isopropanol was then removed with blotting paper and 1ml of stacking gel was pipetted on top of the resolving gel, a 12 well comb introduced then left to set for a further 20 minutes approximately. Gels were made fresh the day prior to running a western blot and stored at $4^{o}C$ overnight saturated in dH_2O. The components of resolving and stacking gels are detailed below in table 2.10.

10% resolution solution for Tris-glycine SDS- polyacrylamide gel	**5% stacking solution for Tris-glycine SDS-Polyacrylamide gel**
To cast one gel (5ml)	
1.9ml distilled water	1.4ml distilled water
1.7ml 30% acrylamide mix	0.33ml 30% acrylamide mix
1.3ml 1.5M Tris (pH 8.8)	0.25ml 1.0M Tris (pH 6.8)
0.05ml 10% SDS	0.01ml 10% SDS
0.05ml 10% ammonium persulfate	0.01ml 10% ammonium persulfate
0.005ml TEMED	0.005ml TEMED

Table 2.10 A table showing the components of resolving and stacking gels used for protein electrophoresis.

2.7.6 Electrophoresis

Gels were loaded into running chambers (mini protean II electrophoresis chamber, Bio-Rad) with lanes facing inward and immersed in running buffer. Prior to loading protein into lanes samples were placed in a warmer at 96^{o}C for 15 minutes then centrifuged to a maximum of 13.3g and placed into ice ready for loading.

5ul of protein ladder was loaded into that far left lane then samples loaded alongside from left to right. Samples were then allowed to run on the gel using 35Amp output for approximately 1.5 hours until the proteins had run down to the bottom of the gel.

Gels were then removed from the glass plates and loaded into a cassette for transfer onto a nitrocellulose membrane abutted by filter paper and sponge either side. Cassettes were then loaded into a transfer chamber and immersed in transfer buffer and run for 30-60 minutes (dependant on the size of chamber) at 110V.

The membrane was then removed and washed in TBS/PBS dependant on the individual antibody protocol.

2.7.7 Immunoblotting

Membranes were blocked in the appropriate blocking agent for 1.5 hours, 10mls per box. This was then exchanged for the primary antibody diluted into 8ml of the appropriate carriage solution and incubated overnight in a cold room ($4^{o}C$).

Primary antibody was then removed and membranes washed in Tris-Buffered Saline (TBS)/PBS for 1 hour changing the wash every 15 minutes.

Species-specific secondary antibody was then introduced and incubated for 1.5 hours. After this a further 1 hour wash with changes every 15 minutes was performed.

2.7.8 Detection

Membranes were immersed in 5ml of Enhanced Chemiluminescence (ECL) detection solution for 5 minutes. This was then removed and membranes blotted dry and loaded into a Kodak light safe exposure cassette and exposed onto medical X-ray film in a dark room and subsequently immersed in Kodak developer then fixative solution, washed and allowed to air dry.

2.7.9 β actin

β-actins were performed on all membranes to control for protein loading. Previously exposed membranes were soaked in stripping buffer (see recipe in table 2.11) then wrapped in clingfilm and left at 75^{o}C for 30 minutes.

Stripping buffer was then removed and 4 15 minute washes in PBS performed. Next blocking in 5% milk was carried out for 1.5 hours and subsequently membranes were immersed in β-actin mouse primary in 5% milk/PBS at the concentration of 1 in 5000 and incubated overnight a 4^{o}C.

Stripping buffer
20ml 10% SDS
12.5ml 1M Tris-HCL pH 6.5
0.7ml β-mercaptoethanol

Table 2.11 A table showing the stripping buffer recipe used.

The primary antibody was then removed and membranes were washed with PBS 4 times, 15 minutes per wash. Secondary anti-mouse Horseradish Peroxidase-Conjugated (HRP) antibody was introduced in 5% milk/PBS at a concentration of 1 in 3000 for 1.5 hrs. A further round of 4 15 minute PBS washes were then performed and detection of the β-actin band using the standard ECL detection method as detailed in section 2.7.8.

2.7.10 Analysis

Western blots were examined using direct visualisation and descriptive comments on up or down regulation made where blots had adequate β-actins on direct visualisation.

CHAPTER 3: MATERIALS AND METHODS *IN VIVO*

A mouse xenograft model with pancreatic cancer cell lines was planned to test AT7519, AT13387 and gemcitabine as single agents and the combination of AT7519 + gemcitabine and AT13387 + gemcitabine. The animal licensee course was undertaken (University of Liverpool) and a personal licence obtained (PIL 40/9684). The experiments where carried out in accordance with project licence PPL40/3320 and all experiments designed under the obligations of the Animals in scientific procedures act set out by the home office with the principles of replacement, reduction and refinement as well as the United Kingdom Co-ordinating Committee on Cancer Research guidelines (UKCCCR)(Workman 1998).

3.1 CELL PREPARATION

3.1.1 Establishing stocks

Pancreatic cancer cell lines were frozen down with adequate stocks to complete all experiments in the xenograft model. As the initial experiment would be establishing the optimum cell line for xenograft studies stocks of SUIT-2 and Miapaca-2 were created. Cells were stored at -150^{o}C.

3.1.2 Restoring cell lines

For each experiment cells used to generate xenografts were prepared in a standardise manner and underwent the same number of passages. The number of vials defrosted depended on the number of tumours required, approximately 1 flask for every tumour required, an excess of 10% was prepared each time. Cells were tested for mycoplasma once growth was re-established.

Vials of cell line were obtained from -150^{o}C stores and underwent a rapid defrost. They were suspended in 10ml of warmed RPMI media and spun in a 15ml falcon tube at 200rpm for 5 minutes. The media was then removed and the cell pellet resuspended into 20ml of RPMI media and incubated in a T75 flask. Cells were incubated for 4 days at 37oC in 5% CO_2 and then split to 1 in 10 in the standard method described in section 2.3.2. The day before planned injections cells

underwent a further split of 1 in 2 to achieve 70-80% confluence on the day of harvest.

On the day of injection cells were prepared. Initially they underwent 2 PBS washes then were trypsinised with 2ml of trypsin per T75 flask. Once trypinsied 6mls of media was added to each flask to make a total volume of 8ml. Flasks were then combined into 50ml falcon tubes and spun at 200 rpm for 5 minutes. The media was then removed from the cell pellet and the cell pellet in each 50ml falcon was resuspended in 20ml of cold PBS and spun at 200 rpm for 5 minutes. Subsequently the PBS was removed and the resultant cell pellet resuspended in 10ml of cold PBS and kept on ice.

Cells were counted using 20µl of the stock into a haemocytometer to make a final concentration of 1x 10^7/ml (this usually required a further spin down at 200rpm for 5 minutes to reach the desired concentration). The resultant stock solution of cells was combined with the same volume of Matrigel HESC Matrix (Bectin Dickinson, Oxford, UK) which had undergone a slow defrost overnight on ice and the mixture gently agitated. The cell suspension was kept on ice and injected as soon as possible after preparation in 200µl aliquots per tumour at 1x 10^6 per tumour.

3.2 MICE

Nude mice were supplied by Charles River UK, BALB/c-Nude mice CAnN.Cg-Foxn1^{nu}/Crl. This strain of immunodeficient mouse was developed through crosses and backcrosses between BalB/caBom-nu and BalB/cannCrj-nu at Charles River Japan in 1985. The homozygous strain of animal lacks a thymus, and is therefore unable to produce T cells and immunodeficient. For all studies male 6-8 weeks old animals were used.

During the experimental protocol animals were given 1 week to acclimatise prior to commencing the study and then ear punched to differentiate between animals (figure 3.1). They were weighed daily and monitored for any changes in behaviour that might indicate poor health. In tolerability studies animals were not dosed with agents if they had >15% weight loss compared with animal weight on day 0 and were culled if they had sustained(72 hrs.) weight loss of >20%. In efficacy studies (and therefore tumour bearing animals) mice were not dosed if >15% weight loss and culled if they had sustained weight loss (72 hrs.) of >15% (see full explanation of reasons for cull below).

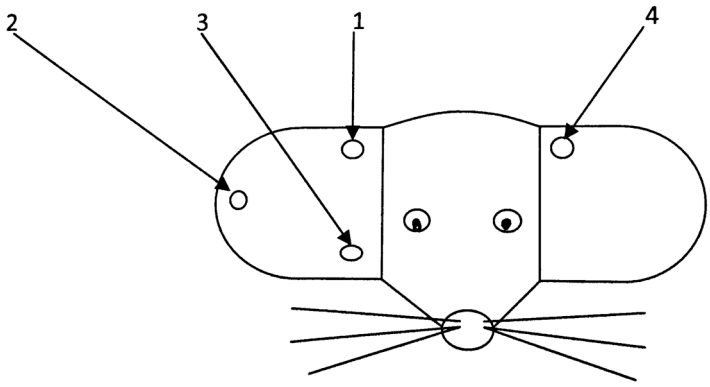


Figure 3.1 A figure showing the rodent ear punch identification map used for experiments.

In the xenograft model tumours were measured thrice weekly with external callipers, taking measurements of length, width and height. Measurements were standardised by being taken by only one individual through all studies and compared with animal handling officer's measurements and compared to ex vivo tumour measurements. Tumour volumes were then calculated using the equation:

(Length x width x height) x 0.5236 = tumour volume

Once the animal was culled they underwent necropsy for organs (heart, lungs, liver, kidneys and spleen) which were suspended in formaldehyde for H & E staining in tumour bearing mice tumours were resected also. The tumours were measured in vivo and ex vivo and weighed ex vivo. The tumours were then divided in half through the midline. The inferior portion was suspended in formaldehyde for

histopathology and the superior portion snap frozen in liquid nitrogen for western blotting.

Animals were culled by a schedule 1 method (CO2 asphyxiation) at the specified experimental end point or when the following circumstances arose:

- Animals demonstrated abnormal behaviour (failure to take food/drink, depressed mobility/lack of interaction)
- Animals developed large ascites (>10% of body weight)
- Large local tumours or distant spread of cancer cells
- Weight loss of 20% body weight sustained over 72 hours
- If the tumour reaches over 17mm diameter or impairs the movement of the animal
- If the tumour starts to ulcerate

3.3 ESTABLISHING THE CELL LINE FOR TUMOUR GROWTH

In order to establish the most appropriate cell line to use for forward experiments a tumour growth experiment was performed. SUIT-2 and Miapaca-2 cell lines were tested. Cell lines were prepared for injection as per the methodology of restoring cell lines detailed in section **3.1.2.** 4 mice per cell line were inoculated with tumours to both left and right shoulders with 200ul of the tumour suspension.

Mice were observed daily and once palpable tumours were established measurements were undertaken of length, width and height of the tumours. The volume of tumours was established as below:

$$(\text{Length} \times \text{width} \times \text{height}) \times 0.5236 = \text{tumour volume}$$

Weight measurements and general well-being observations were also performed daily. The experimental endpoints were tumours reaching the maximum mean diameter of 17mm or 28 days from inoculation with tumours. Post culling the animals ex vivo measurements of tumour volume with callipers, tumour weights and collection of specimens for histopathology were performed.

3.4 PREPARING AGENTS

AT7519 and AT13387 for in vivo studies were provided by Astex Pharmaceuticals, Cambridge, UK. Gemcitabine was bought from LC laboratories MA, USA as Gemcitabine hydrochloride salt (2g, G-4177).

3.4.1 AT7519

AT7519 was provided as the methanesulphonic salt of the compound. Doses are quoted as the base so there was a correction factor applied to derive the correct dose for dilution. The base molecular weight is 493.7 and the salt molecular weight 589.8, therefore the correction factor was 1.19.

Calculations of doses were based on a 20g mouse and enough agent for 7 days treatment was made up on each occasion. One injection at 7.5mg/kg was 0.1875mg per 100ul for the base and 0.223mg per 100ul for the salt. The salt weight was multiplied by the number of injections required and the amount of salt required was established. An excess of 50% was added to this weight to account for losses and the volume of saline required for dilution calculated by dividing this weight by the salt molecular weight multiplied by 100µl. The volume required for each injection was then calculated by multiplying the mouse weight by 5. AT7519 salt was diluted in normal saline. The drug was stored at room temperature as per recommendations

Example:

-calculation based on 20g mouse (6 mice per group, 120 doses in two weeks)

-for one injection 0.15mg per 100ul (for base) = 0.1785mg per 100ul (for salt) (factor 1.19)

- 0.1875 x 120 = 21.42mg (for 120 injections) (in 15ml falcon tube)

-each injection volume 100ul (assuming a 20g mouse)

-to account for loss, need extra 50%, weigh at least 32mg - too much for 15ml falcon tube

Therefore weigh two lots of at least 16mg (two falcon tubes)

-Weight/0.1875 x 0.1ml = volume in ml to dissolve in (saline)

-aliquot 1.5ml per 2ml tube (i.e. for 12 injections – AM and PM separately)

For injections:

-volume per mouse is 5x mouse weight in grams

3.4.2 AT13387

AT13387 was provided as the lactate salt of the compound and the dose of 80mg/kg quoted as the compound base. The base weight is 409.58 and the salt molecular weigh 499.67, thus correction factor was 1.22. One injection at 80mg/kg was 1.6mg per 100µl for the base and 1.95mg per 100µl for the salt. 2-Hydroxypropyl)-β-cyclodextrin powder (Sigma-aldrich, Dorset, UK) was diluted in sterilised water to make a 17.5% solution of cyclodextrin which AT13387 was diluted in. The drug was stored at room temperature as per recommendations.

Example:

-calculation based on 20g mouse (6 mice per group, 2 injections per mouse)

-for one injection 1.6 mg per 100ul (for base) = 1.95 mg per 100ul (for salt) (factor 1.22)

- 1.95 x 12 = 23.4mg (for 12 injections) (in 15ml falcon tube)

-each injection volume 100ul (assuming a 20g mouse)

-to account for loss, need extra 50%, weigh at least 36mg - too much for 15ml falcon tube

Therefore weight two lots of at least 18mg (two falcon tubes)

-Weight/01.95 x 0.1ml = volume in ml to dissolve in (cyclodextrin)

-single aliquot each week

For injections:

-volume per mouse is 5x mouse weight in grams

3.4.3 Gemcitabine

A stock of powered salt was purchased from LC laboratories and diluted in saline. Again a correction against base and salt was required of 1.14 and the calculations made as detailed below. The drug was stored at room temperature as per recommendations.

Example:

-calculation based on 20g mouse (6 mice per group, 24 doses in two weeks)

-for one injection 0.5mg per 100ul (for base) = 0.57mg per 100ul (for salt) (factor 1.14)

- 0.57 x 24 = 13.68mg (for 24 injections) (in 15ml falcon tube)

-each injection volume 100ul (assuming a 20g mouse)

-to account for loss, need extra 50%, weigh at least 21mg

-Weight/0.57 x 0.1ml = volume in ml to dissolve in (saline)

-aliquot: split volume equally in 4 aliquots of at least 1ml each

For injections:

-volume per mouse is 5x mouse weight in grams

3.5 SINGLE AGENT TOLERABILITY EXPERIMENTS

In order to establish the maximum tolerated dose of agents prior to each efficacy study a tolerability experiment was performed. Drug doses for AT13387 and AT7519 tested were established after advice from Astex pharmaceuticals based on previous animal models in other cancers. Doses tested of gemcitabine were based

on current literature. Tolerability experiments were performed in non-tumour bearing mice.

3 mice per group were given intra-peritoneal injections of agents and had daily monitoring of weight and behaviour to establish tolerability. Gemcitabine was tested at 100mg/kg twice per week, AT7519 at 7.5mg/kg twice daily for 5 days and AT13387 at 80mg/kg once weekly; all for 2 weeks (table 3.1). Drugs were deemed tolerated if animals did not experience weight loss or side effects that precluded further dosing or made culling the animal necessary.

	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Dose of gemcitabine	100mg /kg				100mg /kg			100mg /kg				100mg/ kg			

	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Dose of AT7519	7.5mg /kg BD	7.5mg/ kg BD	7.5mg/ kg BD	7.5mg/ kg BD	7.5mg/ kg BD			7.5mg/ kg BD	7.5mg/ kg BD	7.5mg/ kg BD	7.5mg/ kg BD	7.5mg/ kg BD			

	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Dose of AT13387	80mg/ kg							80mg/ kg							

Table 3.1 A table showing the single agent tolerability experiments schedule with the drug dosing for Gemcitabine, AT7519 and AT13387.

3.6 SINGLE AGENT EFFICACY OF GEMCITABINE, AT7519 AND AT13387

Animals were injected with Miapaca2 pancreatic cancer cell lines into the right shoulder as in section **3.1.2**. Once tumours were palpable thrice weekly calliper measurements were undertaken. Drug injections commenced one week after measurements began. Animals were treated for 3 weeks. Each group constituted of six animals and each drug was tested against a vehicle control group. All agents were dosed at the previously determined maximum tolerated dose; Gemcitabine 100mg/kg twice weekly, AT13387 80mg/kg once weekly and AT7519 7.5mg/kg BD for 5 of 7 days. Vehicle control groups included saline BD for 5 of 7 days and cyclodextrin once weekly. Table 3.2 summaries the treatment groups used. The experimental endpoint was tumours reaching the maximum mean diameter of 15mm, weight loss of >20% sustained over 72 hours or completion of treatment (these animals were usually retained for a maximum of 4 days after treatment ceased). Tumour volumes were calculated in excel and regression analysis of tumour growth performed in statview.

Group	Agent	Days of dosing	No of mice
1	Gemcitabine	0 & 4, 7 & 11, 14 & 18	6
2	AT7519	0,1,2,3,4,7,8,9,10,11, 14, 15, 16, 17, 18	6
3	AT13387	1,8, 15	6
4	Saline (Vehicle control of gemcitabine and AT7519)	0,1,2,3,4,7,8,9,10,11, 14, 15, 16, 17, 18	6
5	Cyclodextrin (Vehicle control of AT13387)	1,8, 15	6

Table 3.2 A table showing the single agent efficacy experiments for gemcitabine, AT7519, AT13387 and vehicle controls days of dosing and number of mice used.

3.7 TOLERABILITY COMBINATIONS

In order to establish the maximum tolerated dose of agents prior to each efficacy study a tolerability experiment was performed. Tolerability experiments were performed in non tumour bearing mice. 3 mice per group were given intra-peritoneal injections of agents and had daily monitoring of weight and behaviour to establish tolerability. Agents were deemed tolerated if animals did not experience weight loss or side effects that precluded further dosing or made culling the animal necessary.

AT7519 and gemcitabine were tested in combination with the dose 7.5mg/kg twice daily for 5 days of AT7519 and 50mg/kg twice weekly of gemcitabine (table 3.3).

	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Dose of gemcitabine	50mg/ kg				50mg/ kg			50mg/ kg				50mg/ kg			
Dose of AT7519	7.5mg /kg BD	7.5mg/ kg BD	7.5mg/ kg BD	7.5mg/ kg BD	7.5mg/ kg BD			7.5mg/ kg BD	7.5mg/ kg BD	7.5mg/ kg BD	7.5mg/ kg BD	7.5mg/ kg BD			

Table 3.3 A table demonstrating the dosing regimen for the AT7519 + Gemcitabine tolerability combination experiments.

AT13387 and gemcitabine were tested in combination three times with varying dosing and schedule in order to find the best tolerated experimental design(table 3.4).

	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Dose of gemcitabine	50mg/ kg				50mg/ kg			50mg/ kg				50mg/ kg			
Dose of AT13387		80mg/ kg							80mg/ kg						

	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Dose of gemcitabine	25mg/ kg				25mg/ kg			25mg/ kg				25mg/ kg			
Dose of AT13387		80mg/ kg							80mg/ kg						

	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Dose of gemcitabine	25mg/ kg					25mg/ kg		25mg/ kg					25mg/kg		
Dose of AT13387		80mg/ kg							80mg/ kg						

Table 3.4 A table showing the three tolerability combination experiments performed for AT13387 and gemcitabine.

3.8 COMBINATION EFFICACY STUDIES

In combination efficacy experiments 4 groups of 6 animals each were compared. Theses consisted of one group being given both agents, two groups with one agent and the other agents vehicle control and vice versa and a control group given both agents vehicle controls (table 3.5 and 3.6).

Xenografts were generated as in section 2.1.2 and measurements, observations and endpoints reached as in section 2.3.

Group	Agent	Days of dosing	No of mice
1	Gemcitabine	0 & 4, 7 & 11, 14 & 18	6
	AT7519	0,1,2,3,4 ,7,8,9,10,11, 14,15,16,17,18	
2	Gemcitabine	0 & 4, 7 & 11, 14 & 18	6
	Vehicle control of AT7519	0,1,2,3,4 ,7,8,9,10,11, 14,15,16,17,18	
3	Vehicle control of gemcitabine	0 & 4, 7 & 11, 14 & 18	6
	AT7519	0,1,2,3,4 ,7,8,9,10,11, 14,15,16,17,18	
4	Vehicle control of gemcitabine	0 & 4, 7 & 11, 14 & 18	6
	Vehicle control of AT7519	0,1,2,3,4 ,7,8,9,10,11, 14,15,16,17,18	

Table 3.5 A table showing the dosing regime of the AT7519 + Gemcitabine combination efficacy experiments.

Group	Agent	Days of dosing	No of mice
1	Gemcitabine	0 & 4, 7 & 11, 14 & 18	6
	AT13387	1, 8, 15	
2	Gemcitabine	0 & 4, 7 & 11, 14 & 18	6
	Vehicle control of AT13387	1, 8, 15	
3	Vehicle control of gemcitabine	0 & 4, 7 & 11, 14 & 18	6
	AT13387	1, 8, 15	
4	Vehicle control of gemcitabine	0 & 4, 7 & 11, 14 & 18	6
	Vehicle control of AT13387	1, 8, 15	

Table 3.6 A **table showing the dosing regime of the AT13387 + Gemcitabine combination efficacy experiments.**

3.9 PROCESSING OF ANIMAL TISSUES

3.9.1 H and E staining

Sections of tumour and organs including heart, lungs, liver, kidneys and spleen were extracted from animals at necropsy and immediately fixed in paraformaldehyde. Later these tissue sections were dehydrated further with successive ethanol washes, embedded in paraffin and sectioned with a microtome (by pathologist Dr Fiona Campbell). Sections were stained with haematoxylin **and** eosin and mounted onto glass slides. Histopathology specimens were review by a pathologist (Dr Fiona Campbell).

3.10 STATISTICAL ANALYSIS

Results were maintained on excel spreadsheets and statistical analysis was performed using excel and statsbase. Median tumour volume between treatment groups were compared using Mann Whitney. Differences in the co-efficient of tumour volume were compared between treatment groups using fishers R-Z.

CHAPTER 4: RESULTS *IN VITRO* STUDIES

4.1 TREATMENT OF PANCREATIC CANCER CELL LINES

Initial experiments performed were to establish the ability of agents to inhibit growth of pancreatic cancer cell lines *in vitro*. The results of novel agents AT7519, AT13387 and the standard therapy gemcitabine are included. Inhibition of growth was determined by obtaining IC50 values using an MTS assay. Work using the Fampac cell line was performed by Elisabeth Shaw.

4.1.1 IC50 FOLLOWING TREATMENT WITH GEMCITABINE

The ability of gemcitabine to inhibit growth was assessed in 6 pancreatic cancer cell lines. The half maximal inhibitory concentration (IC50) was determined, as a quantitative measure of how much of the drug was needed to inhibit cell growth by half using MTS assay as a measure of cell viability. Cells were cultured at a seeding density of 1.5×10^4, incubated for 24 hours and then treated with increasing concentrations of Gemcitabine (500nM – 1nM, 1000nM – 5nM for SUIT-2 GR experiments). Cell viability assays were performed at 24, 48 and 72 hours post treatment. Experiments had 6 replicates and were performed in triplicate, growth curves were plotted to determine the IC50.

Table 4.1 shows the IC50 values of gemcitabine for each pancreatic cancer cell line tested. IC50 values ranged from 625nM to 5nM. In a Gemcitabine resistant cell line, SUIT-2 (GR), IC50 values obtained were markedly increased compared with the other cell lines tested confirming this to be a resistant line. Figures 4.1 and 4.2 show the growth curves for the parent line SUIT-2 and gemcitabine resistant line SUIT-2 (GR). Cell photography shown in figure 4.3 again demonstrates the inhibition of cell growth when treated with gemcitabine in comparison to control plates, regardless of replenishment or removal of gemcitabine.

Cell line	**IC50 Gemcitabine**
SUIT-2	10nM
SUIT-2 (GR)	625nM
Miapaca-2	25nM
Panc-1	150nM
CFpac	180nM
FamPac	5.5nM

Table 4.1 A Table showing the anti-proliferative activity of gemcitabine *in vitro*; IC50 values are shown in a range of pancreatic cancer cell lines. The IC50 ranged from 5nM to 625nM.

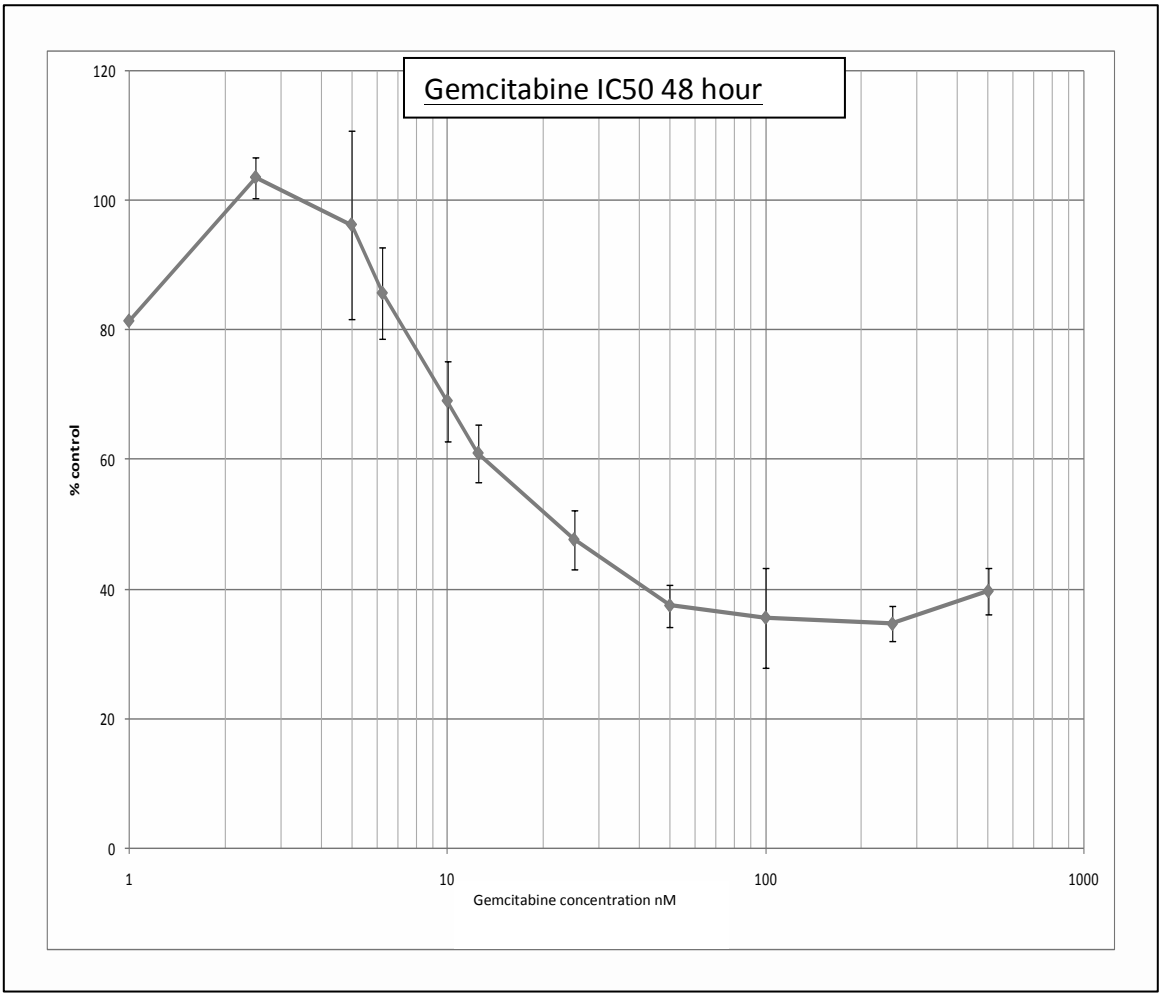


Figure 4.1 A Growth curve of cell viability corrected for controls after treatment with variable concentrations of gemcitabine to establish IC50 in SUIT-2 pancreatic cancer cell line. The X-axis shows the gemcitabine concentration (the plotted data points represent the gemcitabine concentration used), the Y-axis shows the percentage of viable cells compared to controls. MTS assay data at 48 hours is shown.

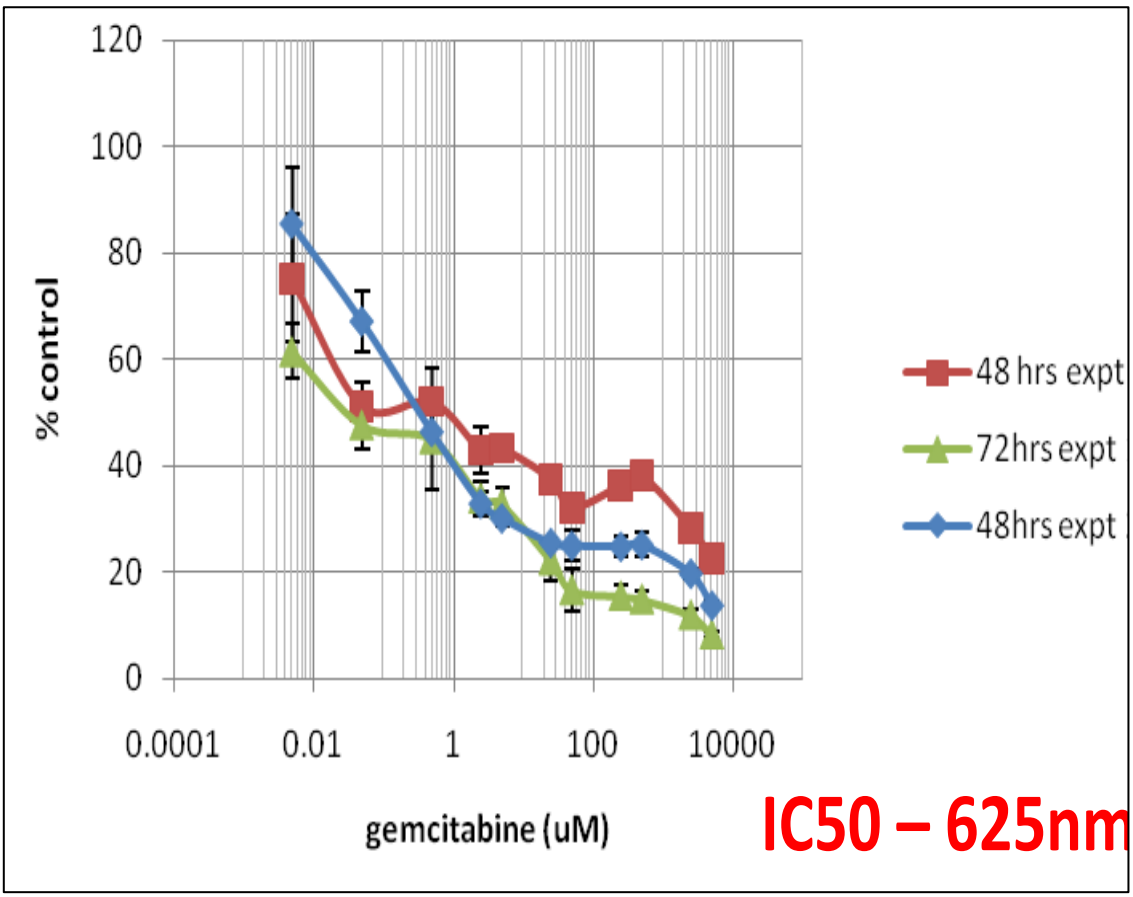
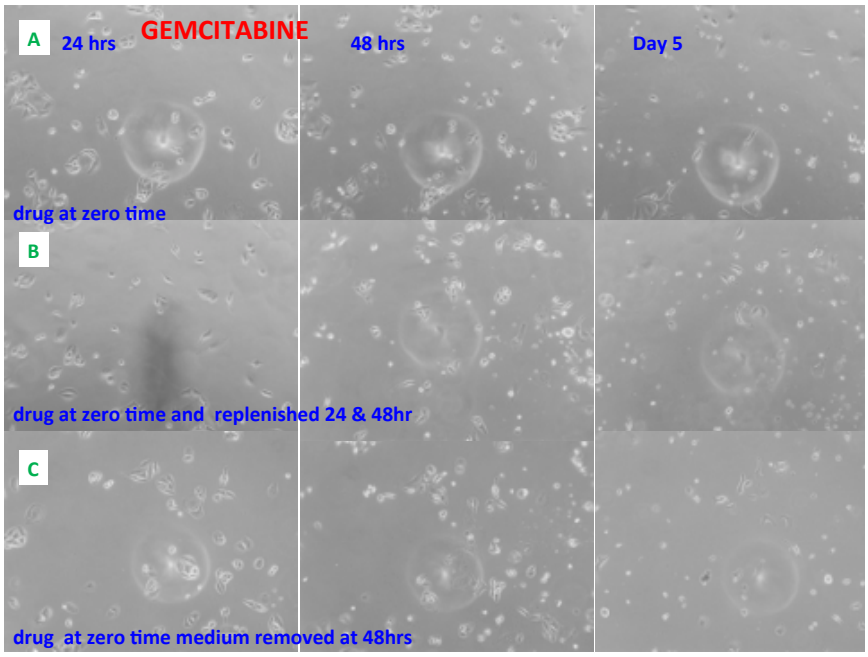


Figure 4.2 A graph showing growth curves of cell viability corrected for controls after treatment with variable concentrations of gemcitabine in SUIT-2 (GR) pancreatic cancer cell line. The X-axis shows the gemcitabine concentration (the plotted data points represent the gemcitabine concentration used, error bars are shown), the Y-axis shows the percentage of viable cells compared to controls. This graph shows two MTS assay experiments with readings taken at 48 hours and one with readings at 72 hours.

[1]



[2]

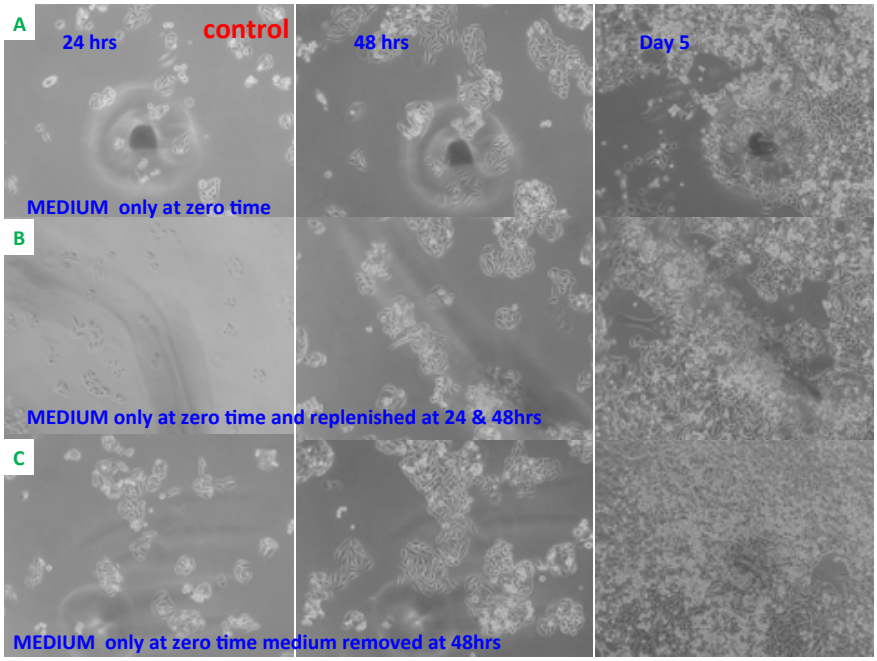


Figure 4.3 Pictures showing cell photography of SUIT-2 cells in 96-well plates at seeding density 1.5 x 10^4 (By Elisabeth Shaw) Cell photography was performed at 24 hours, 48 hours and 5 days.
[1] Treated with 10nM of Gemcitabine [2] In DMSO 0.1% as control
A: drug or control introduced at 0 time point.
B: drug or control introduced at 0 time point then replenished at 24 and 48 hrs.
C: drug or control introduced at 0 time point and then removed at 48 hrs.

CONCLUSION

Gemcitabine was effective in inducing growth inhibition across a range of pancreatic cancer cell lines. The IC50 value for a gemcitabine resistant cell line was 60-fold higher than that of the parent line (625nM in SUIT-2 (GR), 10nM in SUIT-2).

4.1.2 IC50 OF PANCREATIC CANCER CELL LINES FOLLOWING TREATMENT WITH AT7519

The ability of AT7519 to inhibit growth was assessed in 6 pancreatic cancer cell lines. After seeding in 96-well plates cells were cultured with increasing concentrations of AT7519 (50nM-5000nM) and MTS assay was performed on plates at 24, 48 and 72 hours. Experiments had 6 replicates and were performed in triplicate and growth curves plotted to determine the IC50. The 72 hour reading IC50 values are shown in Table 4.2. Growth curves and determined IC50 values are shown in figures 4.4 and 4.5 Cell photography shown in figure 4.6 demonstrates the inhibition of cell growth when treated with AT7519 in comparison to control plates.

The IC50 values obtained ranged from 180nM to 2000nM. The gemcitabine resistant cell line SUIT-2 GR had comparable sensitivity to AT7519 and gemcitabine (table 4.3). SUIT-2 GR was notably more sensitive to treatment with AT7519 than

the parent line SUIT-2 (700nM versus 2μM) the converse was observed after treatment with gemcitabine (625nM versus 10nM).

Cell line	IC50 AT7519
SUIT-2	2μM
SUIT-2 (GR)	700nM
Miapaca-2	390nM
Panc-1	275nM
CFpac	180nM
FamPac	210nM

Table 4.2 A able showing the anti-proliferative activity of AT7519 *in vitro*; IC50 values at 72 hours after treatment with AT7519 are shown in a range of pancreatic cancer cell lines.

Cell line	IC50 AT7519	IC50 Gemcitabine
SUIT-2	2μM	10nM
SUIT-2 (GR)	700nM	625nM

Table 4.3 Table showing the anti-proliferative activity of AT7519 and gemcitabine as single agents *in vitro*; IC50 values are shown for treatment with AT7519 or gemcitabine in pancreatic cancer cell lines SUIT-2 and SUIT-2(GR).

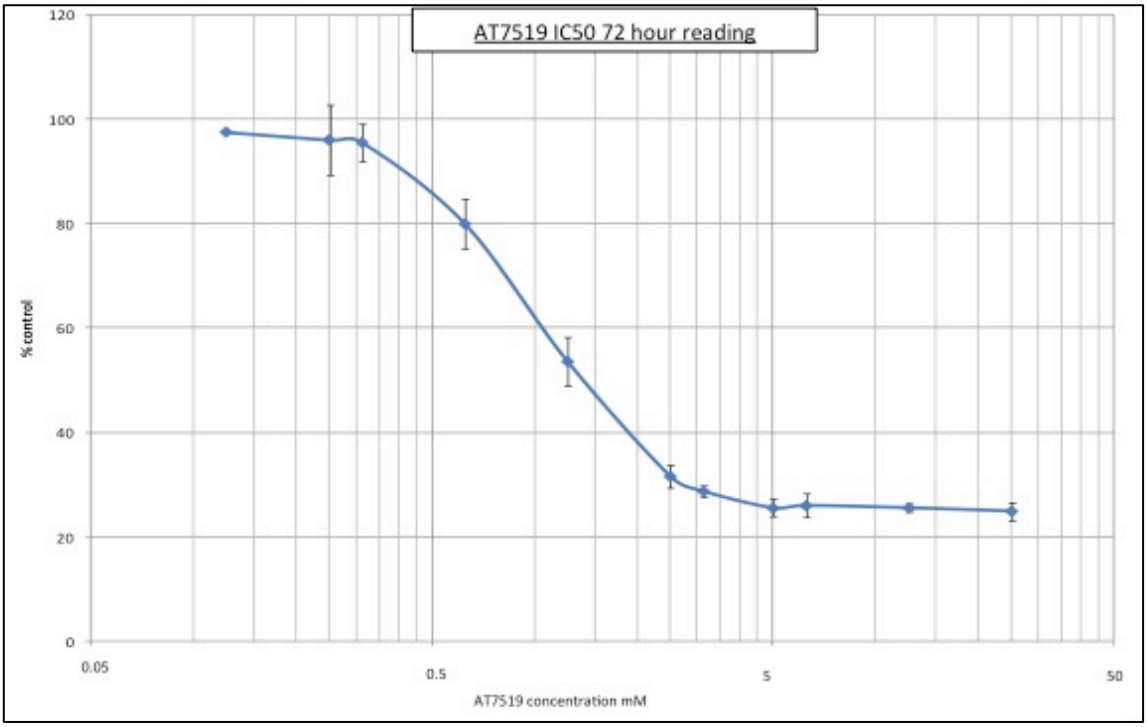


Figure 4.4 Graph showing a growth curve of cell vitality corrected for controls after treatment with variable concentrations of AT7519 in SUIT-2 pancreatic cancer cell line. X-axis shows AT7519 concentration (plotted data points represent AT7519 concentration use, concentration range 250nM - 50mM), Y-axis shows percentage of viable cells compared to controls. The 72 hour reading is shown.

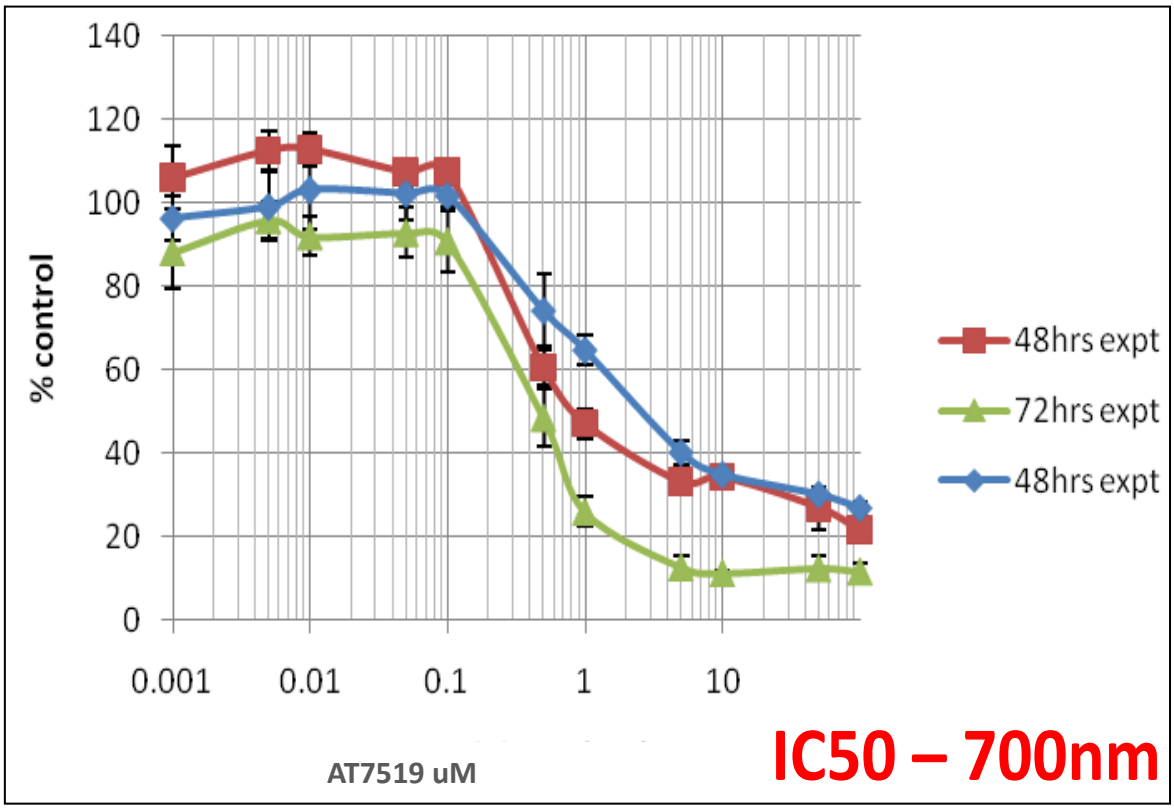


Figure 4.5 Graph showing growth curves of cell viability corrected for controls after treatment with variable concentrations of AT7519 in SUIT-2 (GR). X axis shows AT7519 concentration (plotted data points represent AT7519 concentration used, error bars shown), Y axis shows percentage of viable cells compared to controls. Shows two experiments with readings at 48 hours and one with reading at 72 hours.

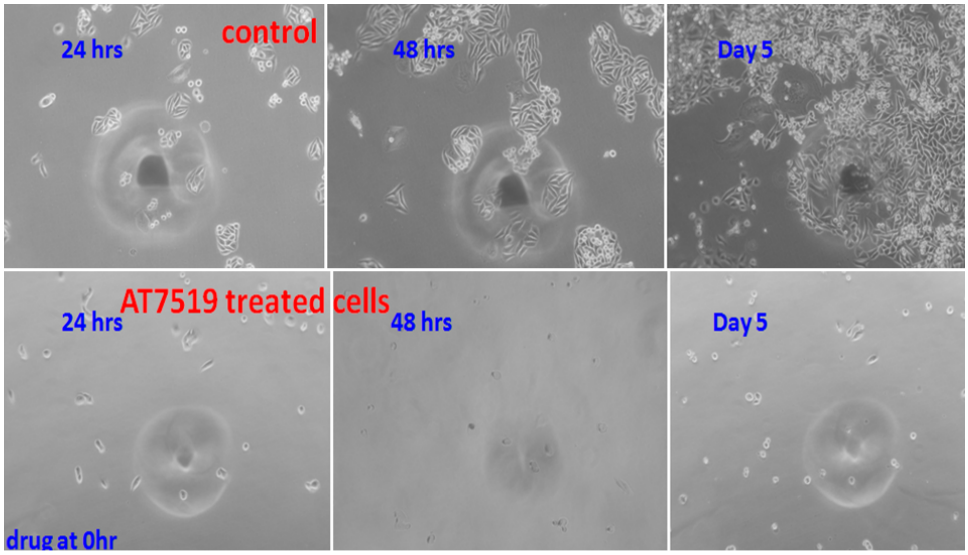


Figure 4.6 Pictures of cell photography of SUIT-2 cells in 96-well plates at seeding density 1.5 x 10^4 (By Elisabeth Shaw) The upper row is control cells left to grow in media with photography at 24, 48 hours and 5 days (media introduced at 0 hours). The lower row shows cells treated with 10µM of AT7519 at 0 hours then cell photography at 24, 48 hours and 5 days.

CONCLUSION

AT7519 inhibited proliferation across a range of pancreatic cancer cell lines with IC50 values ranging from 180nM to 2µM. The gemcitabine resistant line SUIT-2 (GR) was more sensitive than the parent line to AT7519 offering encouraging results to overcome the issue of gemcitabine resistance in pancreatic cancer (IC50 700nM versus 2µM).

4.1.3 IC50 OF AT13387 TREATMENT OF PANCREATIC CANCER CELL LINES

The ability of AT13387 to inhibit growth was assessed in 7 pancreatic cancer cell lines. After seeding in 96-well plates at 1.5 x 10^4 cells were cultured with increasing concentrations of AT13387 (5mM - 5nM) and MTS assay was performed on plates at 24, 48 and 72 hours. The 72 hour reading IC50 values are shown in table 4.4. Growth curves and determined IC50 values are shown in figures 4.7 and 4.8.

The IC50 values obtained ranged from 42nM to 100nM. The gemcitabine resistant cell line SUIT-2 (GR) and the parent line SUIT-2 had comparable sensitivity to AT13387. SUIT-2 (GR) was significantly more sensitive to treatment with AT13387 than gemcitabine. Experiments had 6 replicates and were performed in triplicate and growth curves plotted to determine the IC50. Cell photography shown in figure 4.9 demonstrates the inhibition of cell growth when treated with AT13387 in comparison to control plates. When experiments were performed removing the treatment after 48 hours cell re-growth was observed. This pointed towards a cytostatic rather than cytotoxic effect of AT13387 on the cell lines.

Cell line	IC50 AT13387
SUIT-2	63nM
SUIT-2 (GR)	60nM
Miapaca-2	58nM
Panc-1	50nM
CFpac	100nM
FamPac	42nM

Table 4.4 A table showing the anti-proliferative activity of AT13387 *in vitro*; IC50 values are shown for treatment with AT13387 in a range of pancreatic cancer cell lines. IC50 ranged from 29nM to 325nM.

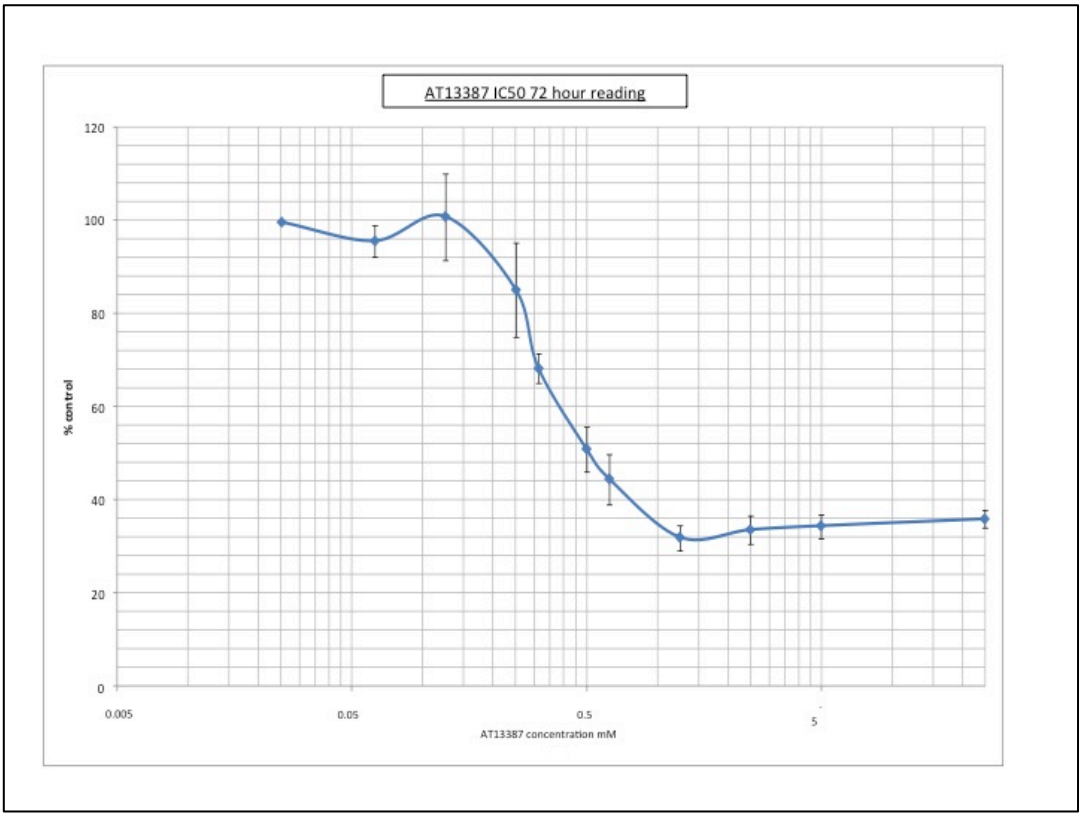


Figure 4.7 A graph showing a growth curve of cell viability corrected for controls after treatment with variable concentrations of AT13387 in SUIT-2 cell line. The 72 hour reading is shown. The X axis shows AT7519 concentration (plotted data points represent AT13387 concentration use, concentration range 5nM - 5mM), the Y axis shows percentage of viable cells compared to controls.

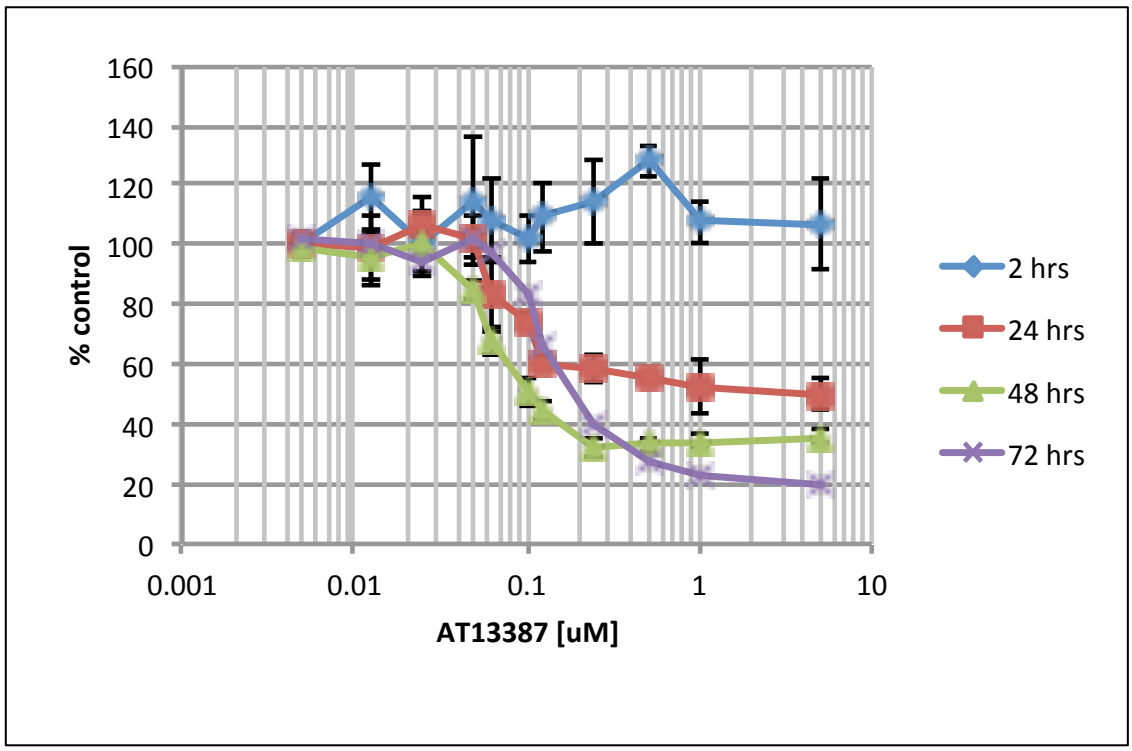
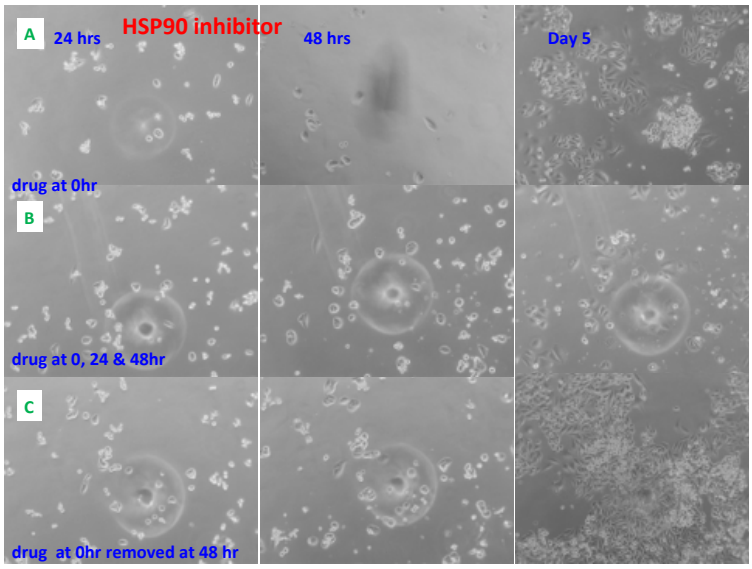


Figure 4.8 A graph of growth curves of cell vitality corrected for controls after treatment with variable concentrations of AT13387 in SUIT-2 (GR) cell line. An MTS assay was performed at 2, 24, 48 and 72 hours. The X axis shows AT13387 concentration (plotted data points represent AT13387 concentration used, error bars shown), the Y axis shows percentage of viable cells compared to controls.

[1]



[2]

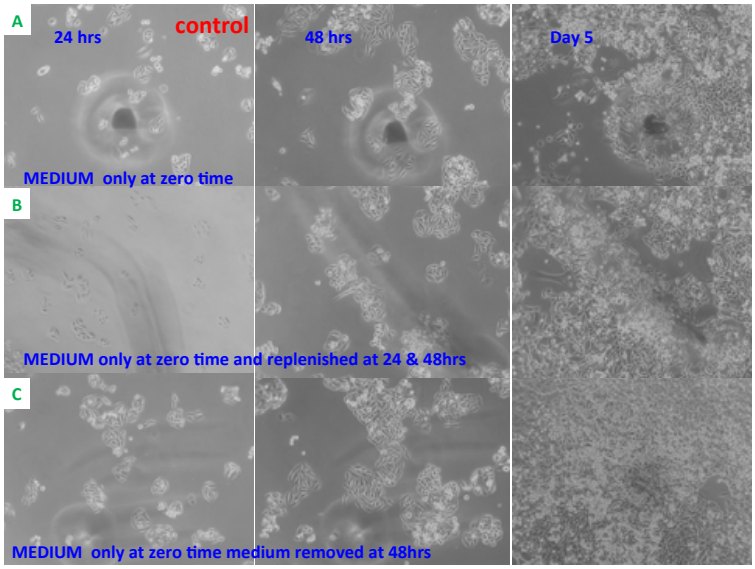


Figure 4.9 Pictures of cell photography (By Elisabeth Shaw), showing SUIT-2 cells in 96-well plates at seeding density 1.5 x 10^4 Cell photography was performed at 24 hours, 48 hours and 5 days.
[1] Treated with 250nM of AT13387 [2] In DMSO 0.1% as control
A: drug or control introduced at 0 time point.
B: drug or control introduced at 0 time point then replenished at 24 and 48 hrs.
C: drug r control introduced at 0 time point and then removed at 48 hrs.

CONCLUSION

AT13387 inhibited proliferation in a range of pancreatic cancer cell lines and the sensitivity of a gemcitabine resistant line SUIT-2 GR was comparable to that of the parent line in contrast with the marked resistance to gemcitabine observed previously. From cell photography a cytostatic rather than cytotoxic effect of AT13387 was observed.

4.2 CELL CYCLE ANALYSIS

In order to examine the effects on the cell cycle from each of these novel agents in pancreatic cancer cell lines cell cycle analysis was performed via propidium iodide (PI) staining and flow cytometry (experiments primarily performed by Elisabeth Shaw with my assistance).

4.2.1 Cell cycle analysis gemcitabine

SUIT-2 cells were seeded onto 6 well plates at 2.5 x 10^5 cells per well in RPMI media and incubated for 24 hours. Gemcitabine 250nM was introduced and cells further incubated for 7, 24 and 48 hours before performing the assay.

Cell cycle analysis showed gemcitabine produced a G1/S phase cell cycle arrest in line with expected action from literature (see figure 4.10 and 4.11).

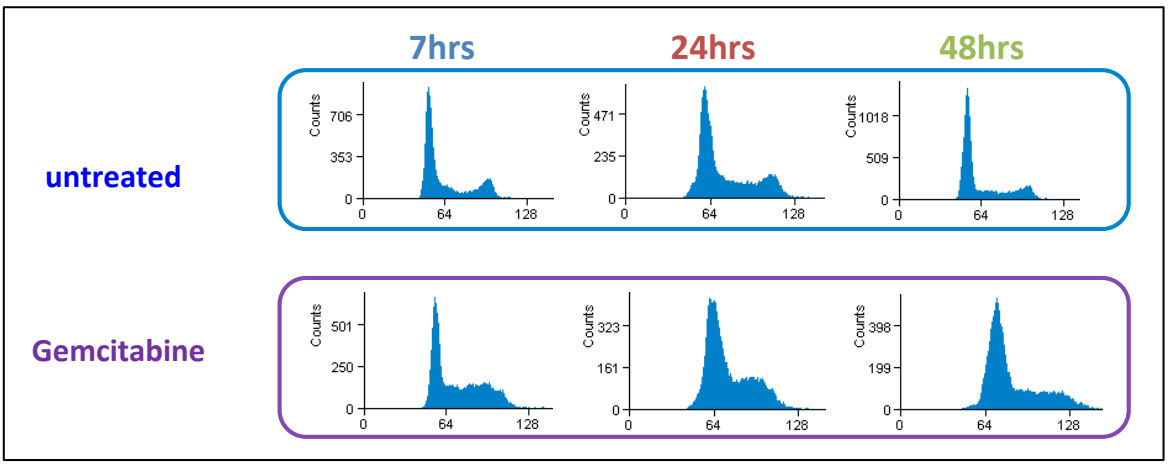


Figure 4.10 A figure showing the cell cycle distribution of SUIT-2 pancreatic cancer cell line untreated (media only) and treated with gemcitabine (250nm) at 7, 24 and 48 hours.

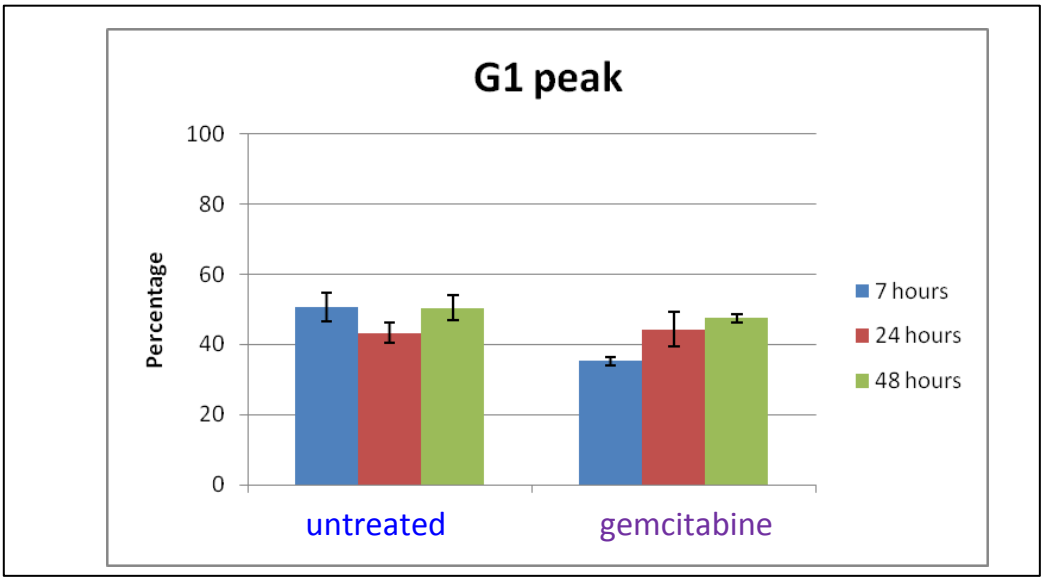


Figure 4.11 Bar chart of percentage of SUIT-2 cells at G1 peak phase of the cell cycle untreated or treated with gemcitabine at 7, 24 and 48 hours. X axis shows cells in groups untreated or treated with gemcitabine (250nM), Y axis shows percentage of cells seen in G1 peak. Legend shows cells at different time point, blue at 7 hours, red at 24 hours and green at 48 hours. Significant difference in the number of cells at G1 peak between untreated and gemcitabine treated cells at 7 hours.

CONCLUSION

Cell cycle analysis showed gemcitabine produced a G1/S phase cell cycle arrest in line with expected action from literature.

4.2.2 Cell cycle analysis AT7519

Suit-2 cells were used to analyze the cytotoxic effects of AT7519 on the cell cycle. Suit2 cells were seeded onto 6 well plates at 2.5 x 10^5 cells per well in RPMI media and incubated for 24 hours before treatment with AT7519 (10μM) or media control. Cell cycle analysis was performed at 4, 7, 24 and 48 hours.

Following 24 hours treatment with AT7519 there was clear arrest in G2/M phase and a reduction in the proportion of cells in G0/G1 and S phase compared to untreated cells ($p=<0.001$) (see figure 4.12, 4.13 and 4.14).

When AT7519 was combined with gemcitabine cell cycle distributions resulted in an accumulation of cells in S phase in keeping with the effects of gemcitabine alone but with a more marked effect than gemcitabine as a single agent (shown in figure 4.15).

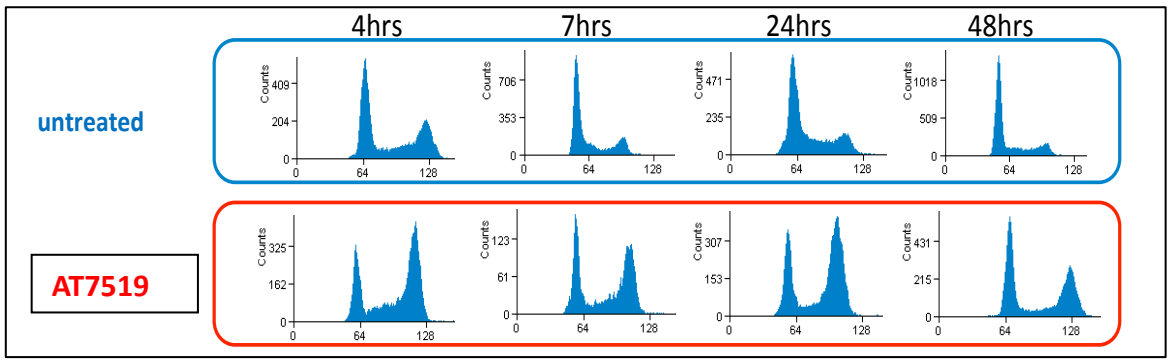


Figure 4.12 A figure showing the cell cycle distribution of SUIT-2 pancreatic cancer cell line untreated (media only) and treated with AT7519 (10µM) at 4, 7, 24 and 48 hours.

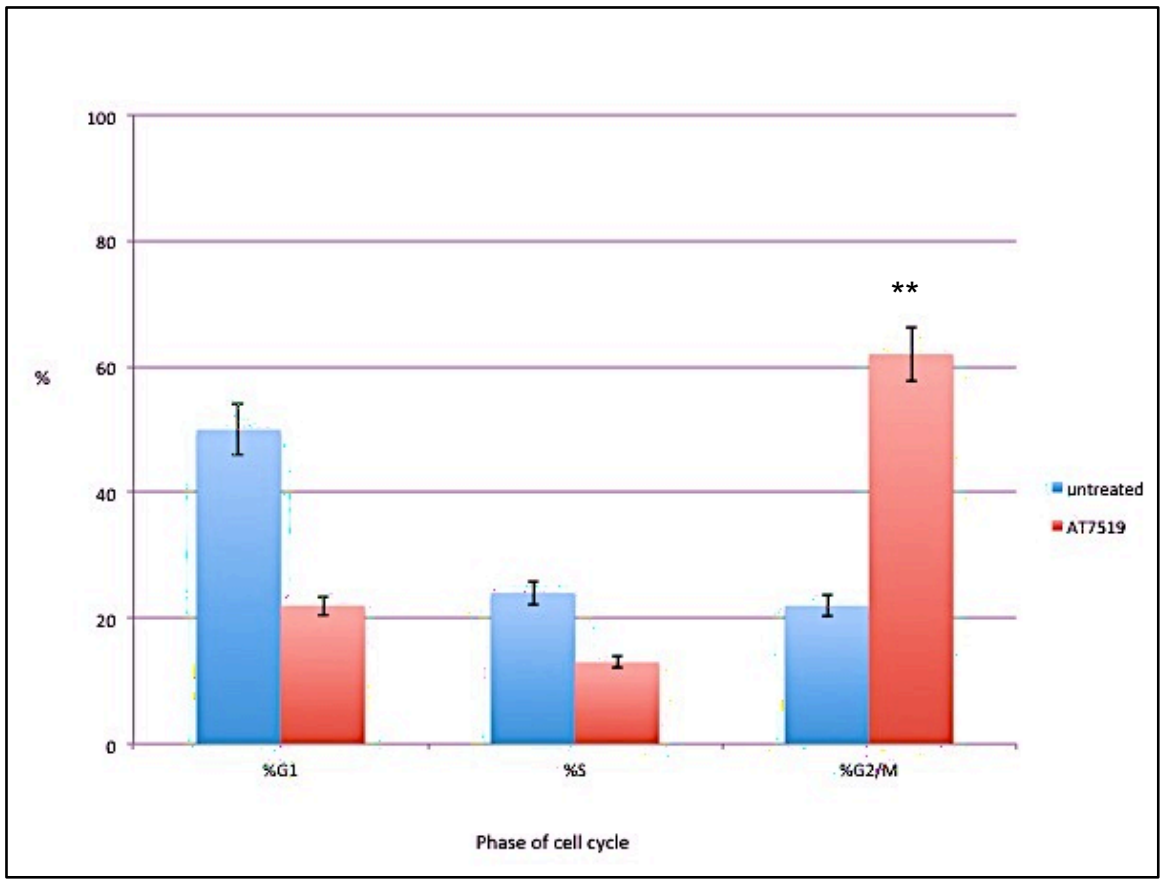


Figure 4.13 A bar chart of percentage of SUIT-2 cells in phases of the cell cycle treated with AT7519 (10µM) versus untreated control cells 7 hours after drug addition. The X-axis shows phase of cell cycle G1, S phase and G2/M, the Y-axis represents percentage of cells seen in each phase. ** p<0.01.

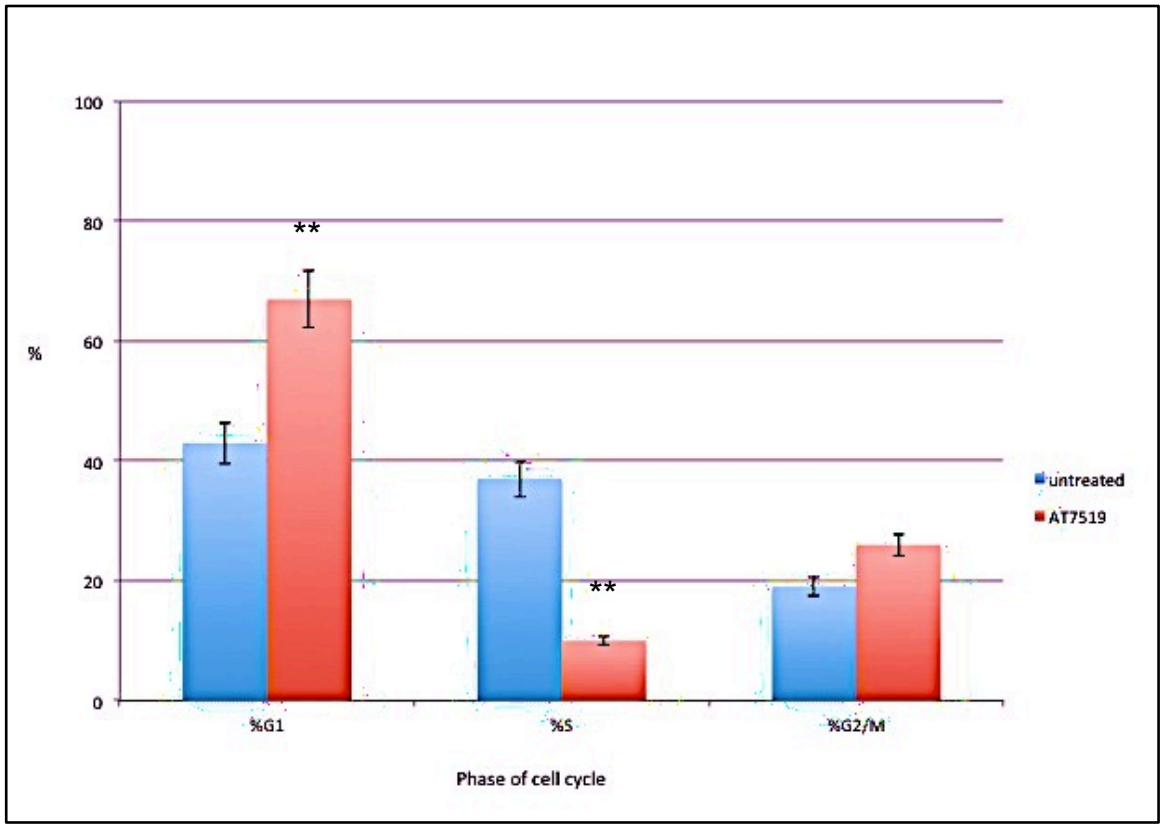


Figure 4.14 A bar chart of percentage of SUIT-2 cells in different phases of the cell cycle treated with AT7519 (10µM) versus untreated control cells 24 hours after drug addition. The x-axis shows phase of cell cycle G1, S phase and G2/M, the y-axis represents percentage of cells seen in each phase. ** p<0.01.

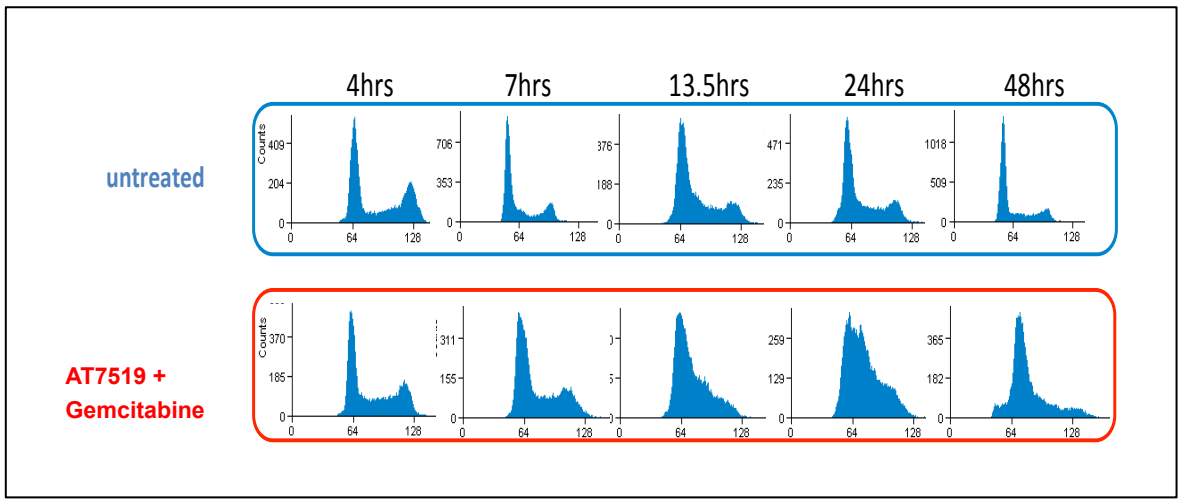


Figure 4.15 A figure showing the cell cycle distribution of SUIT-2 pancreatic cancer cell line untreated (media only) and treated with AT7519 (2µM) + Gemcitabine (10nm) at 4, 7, 13.5, 24 and 48 hours.

CONCLUSION

Cell cycle analysis showed a marked reduction of cells in the G0/G1 and S phases with an increase in cells in G2/M phase following treatment with AT7519. When AT7519 was combined with gemcitabine the effects observed were in line with that of gemcitabine alone with an accumulation of cells in S phase.

4.2.3 Cell cycle analysis AT13387

The effect of AT13387 treatment on cell cycle progression was assessed in SUIT-2 cells. SUIT-2 cells were seeded onto 6 well plates at 2.5 x 10^5 cells per well in RPMI media and incubated for 24 hours before treatment with AT13387 (250nM) or media control. Cell cycle analysis was performed at 4, 7, 24 and 48 hours.

Following 24hrs of treatment with AT13387 cells showed a significant increase in cells in the G0/G1 ($P<0.01$) and G2/M ($P<0.05$) phases of the cell cycle compared to vehicle treated controls (figure 4.16 and 4.17).

Cells treated with both AT13387 and Gemcitabine showed a marked difference to single drug treatment with an increase in cells in the s-phase of the cell cycle in keeping with the effects of Gemcitabine alone but more perturbed (figure 4.18).

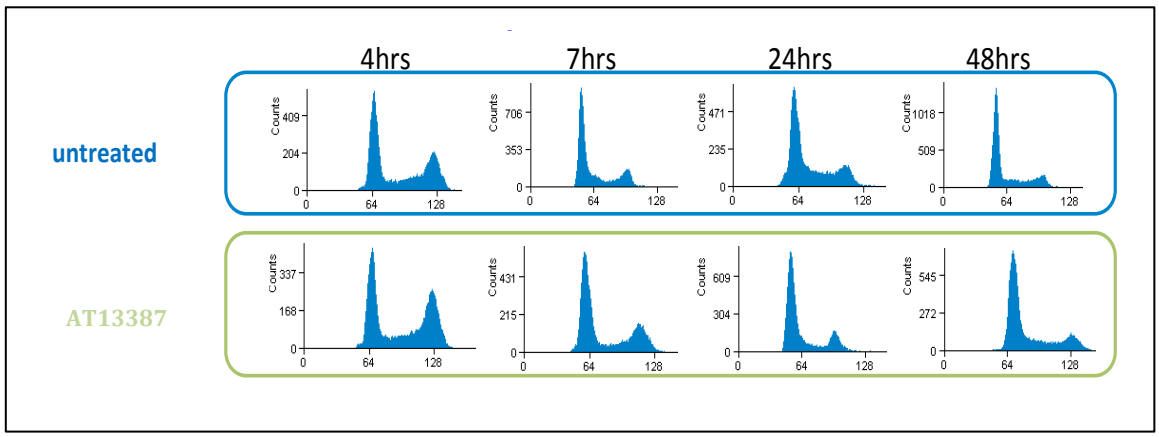


Figure 4.16 A figure showing the cell cycle distribution of SUIT-2 pancreatic cancer cell line untreated (media only) and treated with AT13387 (250nm) at 4, 7, 24 and 48 hours.

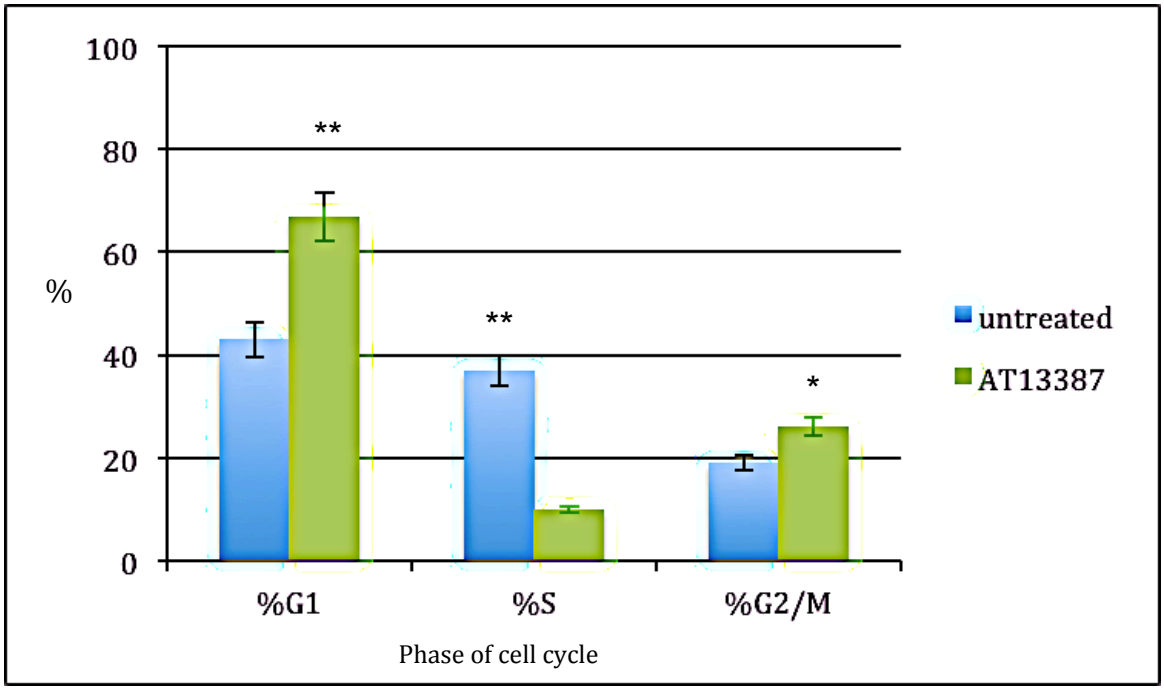


Figure 4.17 A bar chart of the percentage of cells in different phases of the cell cycle treated with AT13387 versus untreated control SUIT-2 cells at 24 hour time point. The x-axis shows phase of cell cycle G1, S phase and G2/M, the y-axis represents percentage of cells seen in each phase. * p<0.05. ** p<0.01.

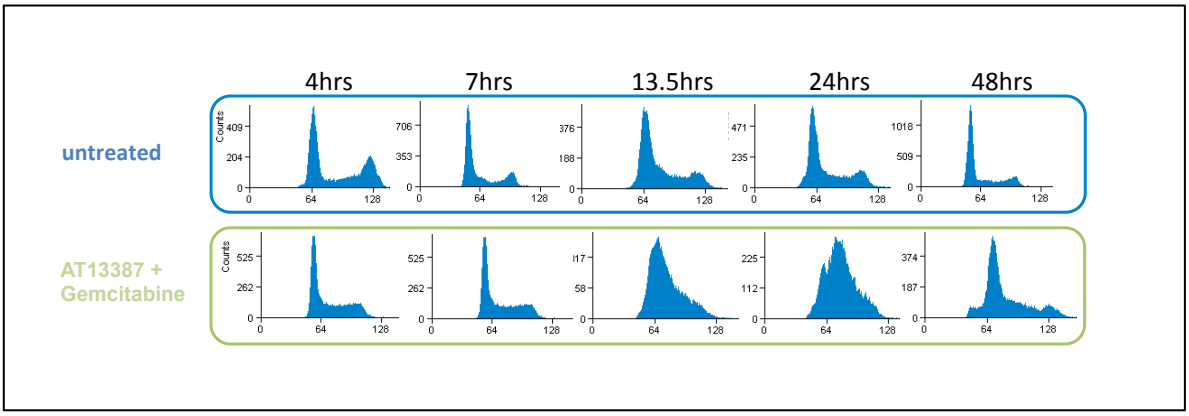


Figure 4.18 A figure showing the cell cycle distribution of SUIT-2 pancreatic cancer cell line untreated (media only) and treated with AT13387 (63nM) + Gemcitabine (10nM) at 4, 7, 13.5, 24 and 48 hours.

CONCLUSION

AT13387 treatment leads to cell cycle arrest in G0/G1 and G2/M phases of cell cycle. In combination with gemcitabine AT13387 showed S phase accumulation in line with gemcitabine action but more marked.

4.3 APOPTOSIS ASSAYS

Experiments in this section were performed by Elisabeth Shaw.

4.3.1 Apoptosis assays gemcitabine

Evidence of induction of apoptosis by gemcitabine was tested in Miapaca-2 pancreatic cancer cell line by investigation of caspase-3 activity. The caspase-Glo 3/7 luminescent assay was performed on cells treated with 250nM of Gemcitabine or 0.1% DMSO control at 6, 16, 24, 40, 48 and 72 hours. At 40, 48 and 72 hours induction of caspase 3/7 was significantly increased in gemcitabine treated cells compared with those in control media ($p=0.01$) (figure 4.19).

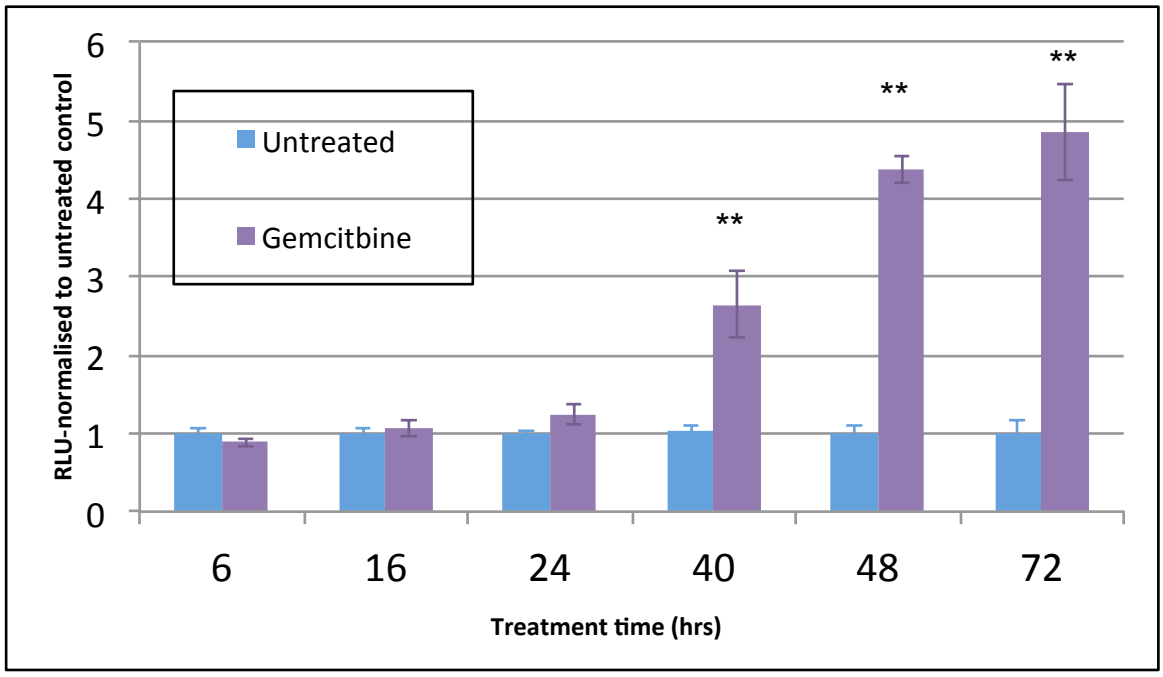


Figure 4.19 A bar chart showing caspase-3/7 activity of untreated and gemcitabine (250nM) treated Miapaca-2 pancreatic cancer cells at 6, 16, 24, 40, 48 and 72 hours. The x-axis shows the time point of the assay and the y-axis shows Relative Light Units (RLU)-normalised to untreated control cells. ** p=0.01.

CONCLUSION

Apoptosis was demonstrated after treatment with gemcitabine in vitro by the surrogate marker caspase-3.

4.3.2 Apoptosis assays AT7519

Evidence of induction of apoptosis by AT7519 was tested in Miapaca-2 pancreatic cancer cell line by investigation of caspase-3 activity. The caspase-Glo 3/7 luminescent assay was performed on cells treated with 10µM of AT7519 or 0.1% DMSO control at 6, 16, 24, 40, 48 and 72 hours.

At 24 hours induction of caspase 3/7 was significantly increased in AT7519 treated cells compared with those in control media (p=0.01), upregulation of caspase 3/7 was also noted at 16 and 40 hour time points (figure 4.20).

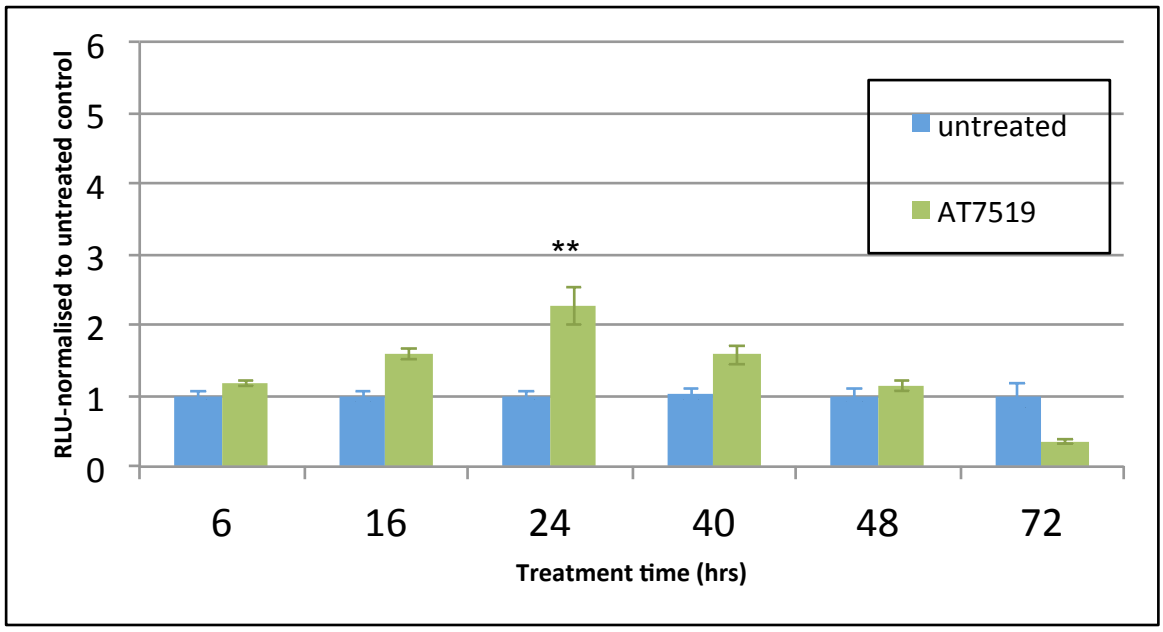


Figure 4.20 A bar chart showing the caspase-3/7 activity of untreated and AT7519 (10µM) treated Miapaca-2 pancreatic cancer cells at 6, 16, 24, 40, 48 and 72 hours. The x-axis shows time point of assay and the y-axis shows RLU-normalised to untreated control cells. ** p=0.01

CONCLUSION

Apoptosis was demonstrated after treatment with AT7519 in vitro by the surrogate marker caspase-3.

4.3.3 Apoptosis assays AT13387

Treatment of AT13387 had already proven inhibition of growth and cell cycle arrest but this does not necessarily equate to cell death therefore investigation was undertaken to establish if apoptosis was occurring using caspase 3/7 activity as a marker of this. After treating Miapaca-2 cells with 1µM of AT13387 or 0.1% DMSO control the caspase-glo 3/7 luminescent assay was performed at time points including 6, 16, 24,40,48 and 72 hours (experiment performed by ES). At 40, 48 and 72 hours there was a substantial increase in caspase 3/7 activity (figure 4.21).

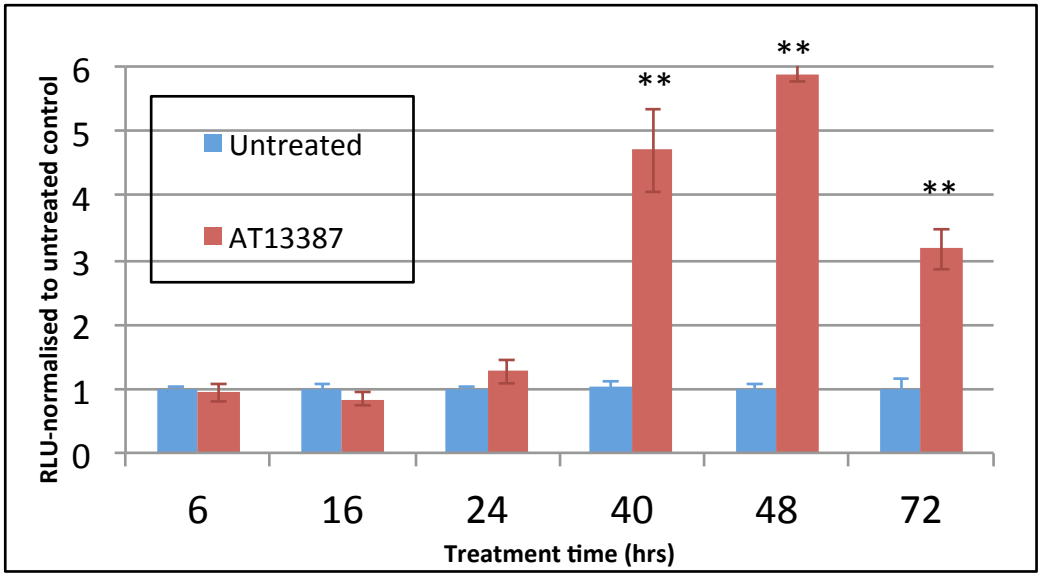


Figure 4.21 A bar chart showing the caspase-3 activity of untreated and AT13387(1µM) treated Miapaca-2 pancreatic cancer cells at 6, 16, 24, 40, 48 and 72 hours. The x-axis shows time point of assay after treatment and the y-axis shows RLU-normalised to untreated control cells. ** p<0.01.

This observed evidence of apoptosis was further confirmed by the presence of cleaved PARP in Miapaca-2 cells at 30 and 48 hours after treatment , it was noted that cleaved PARP was evident in both adherent and floating cell populations(experiment performed by ES, data not shown).

CONCLUSION

The results from caspase 3/7 assays and evidence of cleaved PARP support the motion that AT13387 resulted in apoptosis of treated pancreatic cells.

4.4 ISOBOLAR ANALYSIS

After establishing the IC50 values for gemcitabine, AT7519 and AT1387 as single agents the combinations of gemcitabine + AT7519 and gemcitabine + AT13387 were investigated using an isobolar analysis. In this experiment cells were plated then treated with both agents at variable concentrations to observe any changes to the established IC50's of each agent in line with synergy or antagonism when the agents were used in combination.

4.4.1 Isobolar analysis of AT7519 and gemcitabine

Cells were seeded in 96-well plates at 1.5 x 10^4 after 24 hours culture fresh media was introduced with variable concentrations of gemcitabine (20nM-2.5nM) and AT7519 (2μM – 0.25μM) with wells of combined agents, single agents and media controls. MTS assays were performed at 24, 48, 36, 72 and 96 hours. IC50 experiments were performed in triplicate with 4 plates per experimental run.

When gemcitabine and AT7519 were added in combination the resulting isobolar curve generated showed an antagonistic effect. These initial results showed AT7519 still had an effect at its recognized IC50 regardless of the presence of gemcitabine however Gemcitabine was inhibited from reaching its IC50 (figure 4.22).

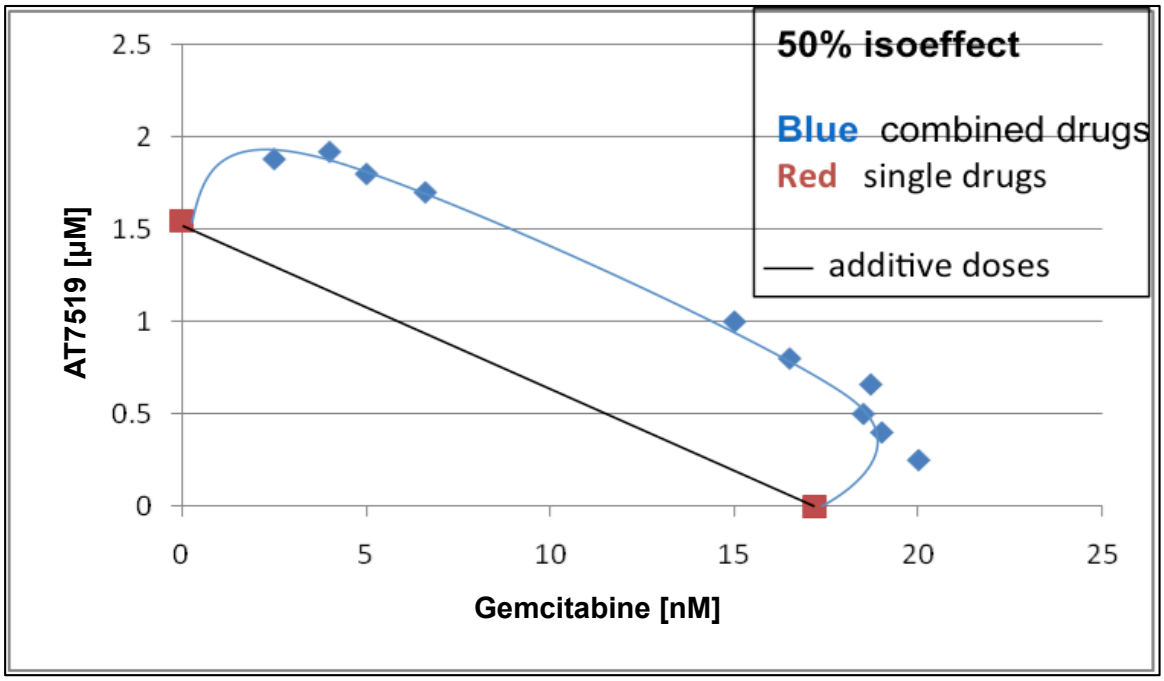


Figure 4.22 A graph showing an isobolar analysis of the combination of AT7519 and gemcitabine. Data is shown from 6 96-well plates representing the average of 50% point of cell growth across plates adjusted for controls. The AT7519 concentration used ranged from 2-0.25 µM and gemcitabine was 20-2nM. Both agents were added to plates at the same time point. The x-axis shows gemcitabine concentration in nM and the y-axis shows AT7519 concentration in µM. Plotted red and blue points represent the 50% isoeffect i.e. the amount of each agent required to produce a 50% cell growth inhibition. Points plotted along the black line would represent an additive effect, points above the line suggest antagonism and below the line represent synergy.

In light of this results further experiments were performed using sequential drug addition. From data generated after discussion it was decided that the addition of gemcitabine for 24 hours followed by combination therapy would be a rational dosing regimen owing to the fact that cell cycle analysis demonstrated cells need to be dividing in order for gemcitabine to have an effect.

Following this sequential addition of drugs and isobolar curve was generated showing at least an additive effect of gemcitabine and AT7519 (figure 4.23). No results were obtained demonstrating synergy.

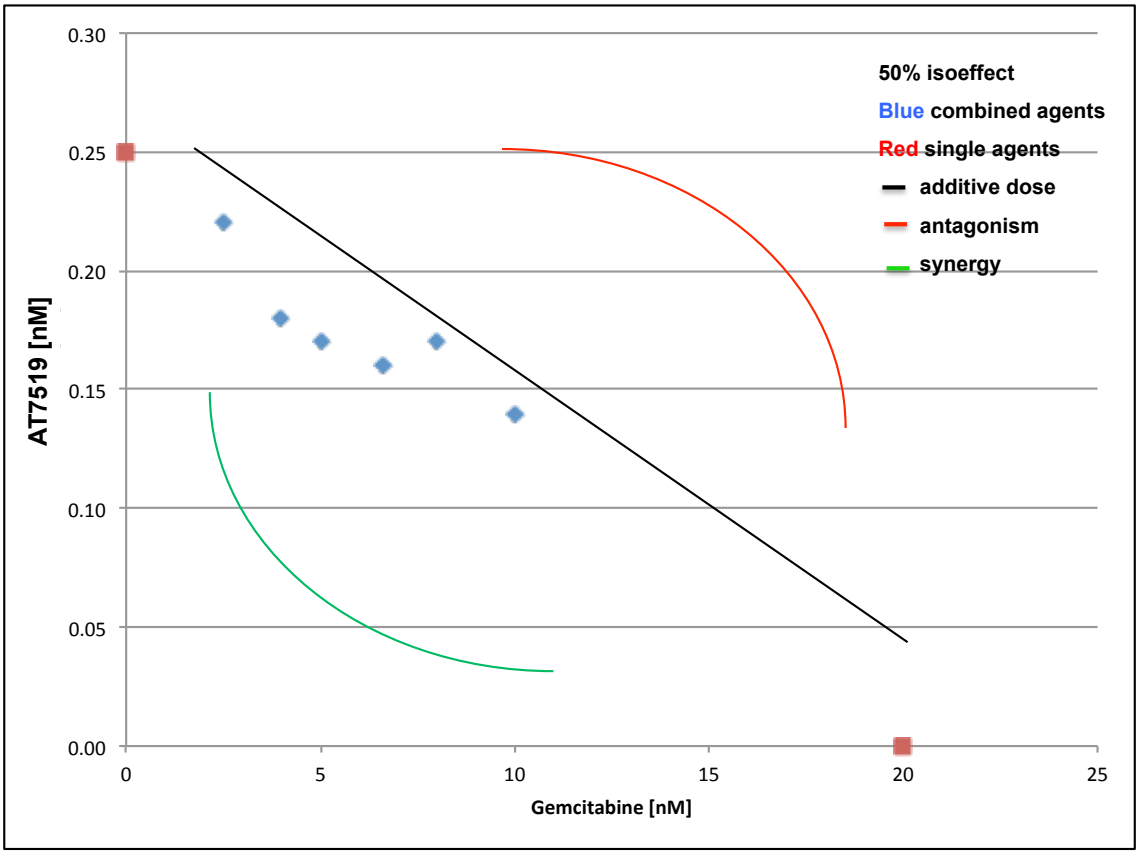


Figure 4.23 A graph showing an isobolar analysis of the combination of AT7519 and gemcitabine when the drugs were added sequentially (gemcitabine treatment for 24 hours then the combination of gemcitabine and AT7519 for 48 hours). Data from 6 96-well plates representing the average of 50% point of cell growth across plates adjusted for controls is shown. The AT7519 concentration used was 2-0.25 µM and gemcitabine concentrations used were 20-2nM. The x-axis shows gemcitabine concentration in nM and the y-axis shows AT7519 concentration in µM. Plotted red and blue points represent the 50% isoeffect i.e. the amount of each agent required to produce a 50% cell growth inhibition. Points plotted along the black line would represent an additive effect, red line above shows antagonism and green below demonstrates synergy.

CONCLUSION

In vitro the combination of gemcitabine and AT7519 had an antagonistic effect on isobolar analysis, however when the scheduling of the agents was modified to sequential addition of gemcitabine then the combination of gemcitabine and AT7519 an additive effect was observed. This supports the cell cycle data observes that demonstrates cells need to be dividing in order for gemcitabine to have an effect. The observation of at least an additive effect supports further investigation of this agent in combination with gemcitabine.

4.4.2 Isobolar analysis of AT13387 and gemcitabine

Cells were seeded in 96-well plates at 1.5 x 10^4 cells per well and cultured for 24 hours, fresh media was then introduced with variable concentrations of gemcitabine (2nM- 40nM) and AT13387 (15nM – 125nM). MTS assays were performed at 48 hours. IC50 experiments were performed in triplicate with 6 plates per run.

The combination of AT13387 and gemcitabine did not yield an adequate isobolar analysis. In one run of the experiment gemcitabine did not have a significant effect as a single agent and in 2 runs AT13387 did not have an effect as a single agent, this made isobolar interpretation impossible. The results were examined in detail and

some observations were made despite this. In the presence of gemcitabine there was a suggestion of enhanced AT13387 activity in one run of this experiment where AT13387 did not reach its IC50 as a single agent. This was observed by calculating the coefficients of the gradients produced but this data was too extrapolated to give a definitive result to comment upon regarding these agents in combination.

CONCLUSION

Isobolar analysis was not completed with AT13387 and gemcitabine but there was a trend that AT13387 activity was enhanced by the presence of gemcitabine.

4.5 WESTERN BLOTTING

4.5.1 Western blotting of downstream targets AT7519

In order to establish the downstream targets/effects of treatment with AT7519 western blotting was performed on a number of downstream targets both to demonstrate activity and to establish any potential biomarkers for the AT7519. A summary of the effects on protein expression are summarized in table 4.5 at the end of this section.

4.5.1.1 cdc2 (CDK1) protein expression

Entry of cells into mitosis is regulated by cdc2 kinase activation. Cell cycle analysis supports an AT7519 G2/M block (as expected if cdc2 activity is inhibited). Western blot analysis was undertaken to examine cdc2 activity after various treatments.

After treatment with AT7519 as a single agent (dose 1µM-10µM) western blot analysis showed no change in cdc2 levels, supporting the notion that activity and not levels of CDK1 are affected by AT7519 (figure 4.24 and 4.25).

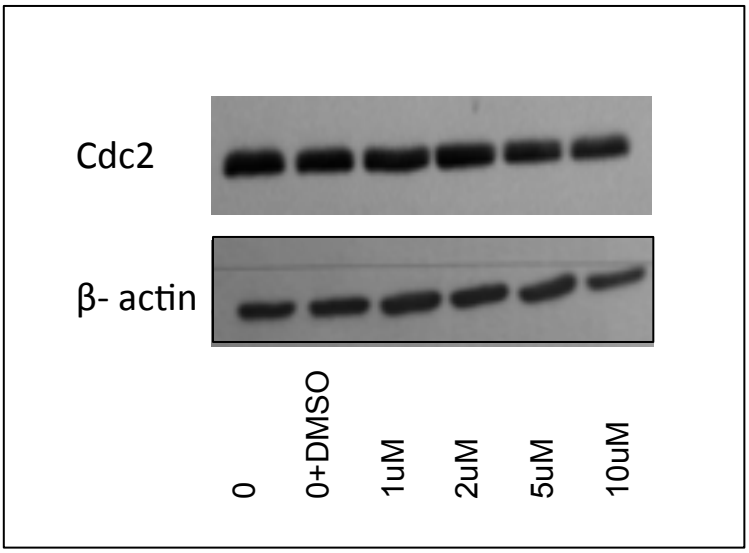


Figure 4.24 A figure showing the western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT7519 (1µM- 10µM) media only or DMSO control for 24 hours. The blots were probed with cdc2 (CDK1) antibody.

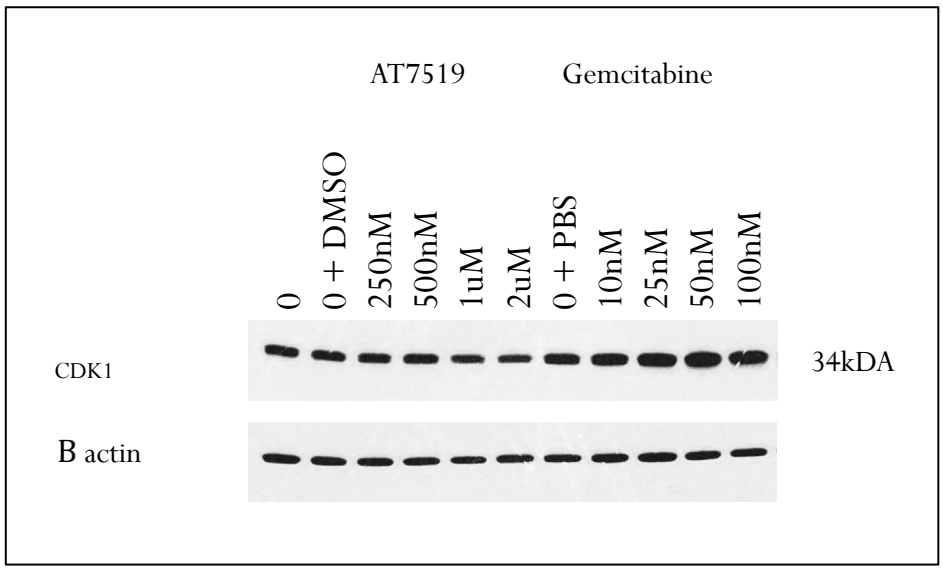


Figure 4.25 A figure showing a western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT7519 (250nM – 2µM) or Gemcitabine (10nM-100nM) or vehicle control. The blots were probed with cdc2 (CDK1) antibody.

When gemcitabine and AT7519 were used in combination this decrease in cdc2 was again observed suggesting AT7519 was having an effect but not gemcitabine. This was confirmed by sequential addition of gemcitabine alone for 24 hours followed by variable concentrations of AT7519, cdc2 levels remained unchanged supporting the observation that gemcitabine was causing an s phase accumulation and the notion that cells need to be dividing in order for a G2/M block to occur (figure 4.26 and 4.27).

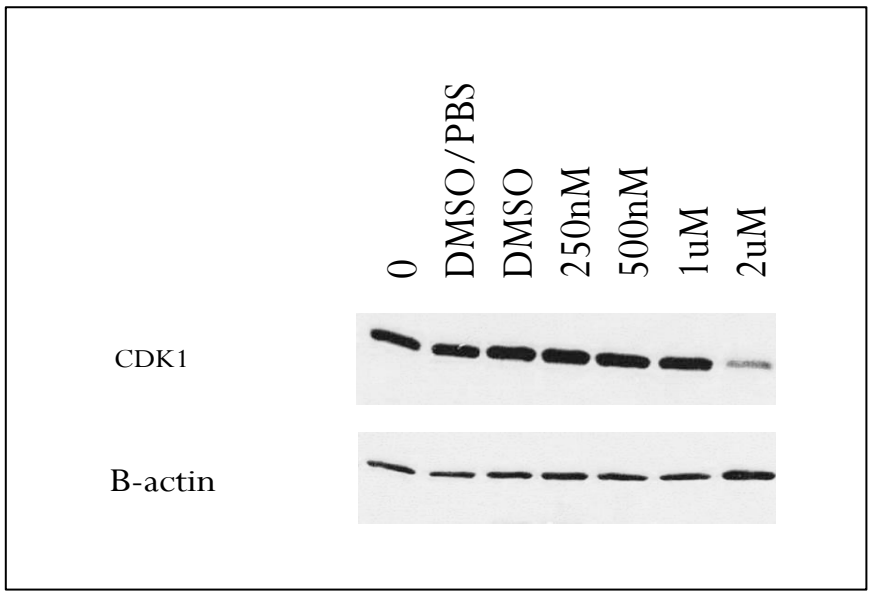


Figure 4.26 A figure showing combination treatment- a western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT7519 (250nM – 2µM) and gemcitabine (25nM) for 24 hours. The blots were probed with cdc2 (CDK1) antibody.

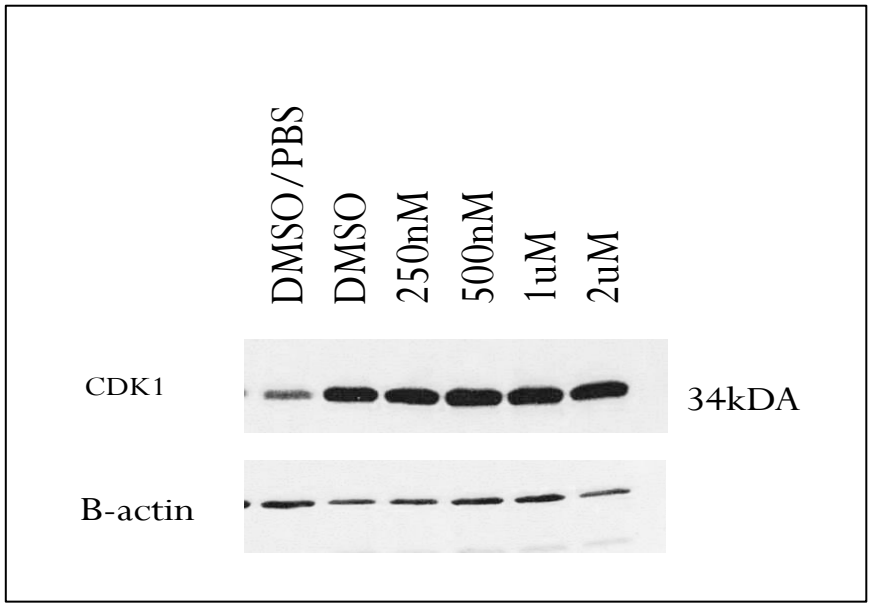


Figure 4.27 A figure showing sequential addition- a western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with gemcitabine (25nM) + AT7519 (250nM – 2µM). Gemcitabine was given for 24 hours then AT7519 for 24 hours. The blot was probed with cdc2 (CDK1) antibody.

CONCLUSION

After treatment with AT7519 as a single agent levels of cdc2 activity were unchanged. In combination AT7519 and gemcitabine displayed a cdc2 decrease, when gemcitabine was given first cdc2 levels were unchanged supporting the notion that cells need to be dividing in order for a G2/M block to occur.

4.5.1.2 Rb/Phospho Rb protein expression

The retinoblastoma protein (Rb) is a tumour suppressor protein that is known to be dysfunctional in cancer. Rb prevents excessive cell growth by inhibiting cell cycle progression from G1 to S phase until a cell is ready to divide at which point it is phosphorylated. Rb phosphorylation is catalysed by cyclin-dependent kinases and therefore it was hypothesized potentially inhibited by AT7519.

From western blots performed Rb phosphorylation with increasing concentrations of AT7519 is almost eliminated despite minimal changes in Rb levels after drug treatment (figure 4.28 and 4.29).

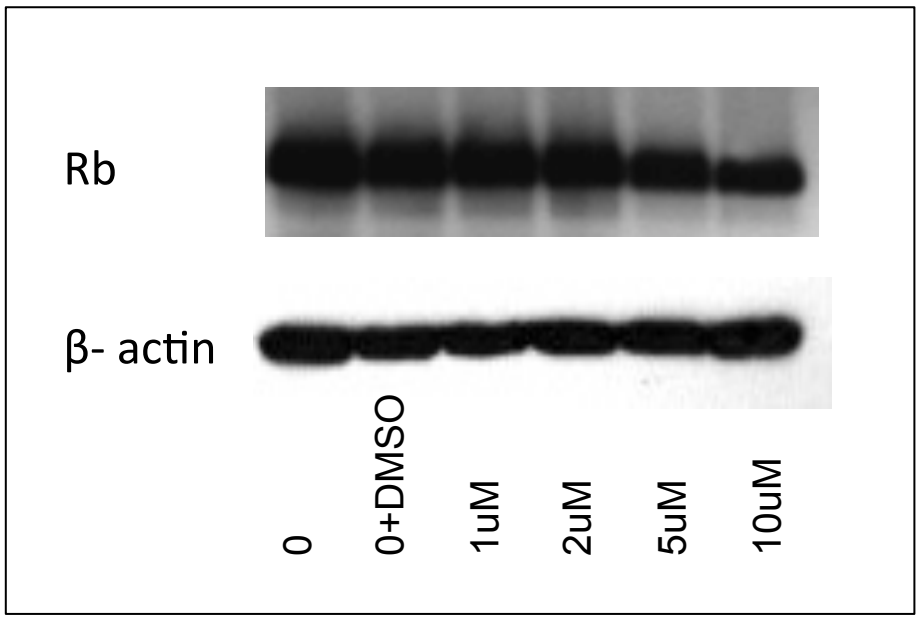


Figure 4.28 A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT7519 (1μM- 10μM) media only or DMSO control for 24 hours. The blot was probed with Rb antibody.

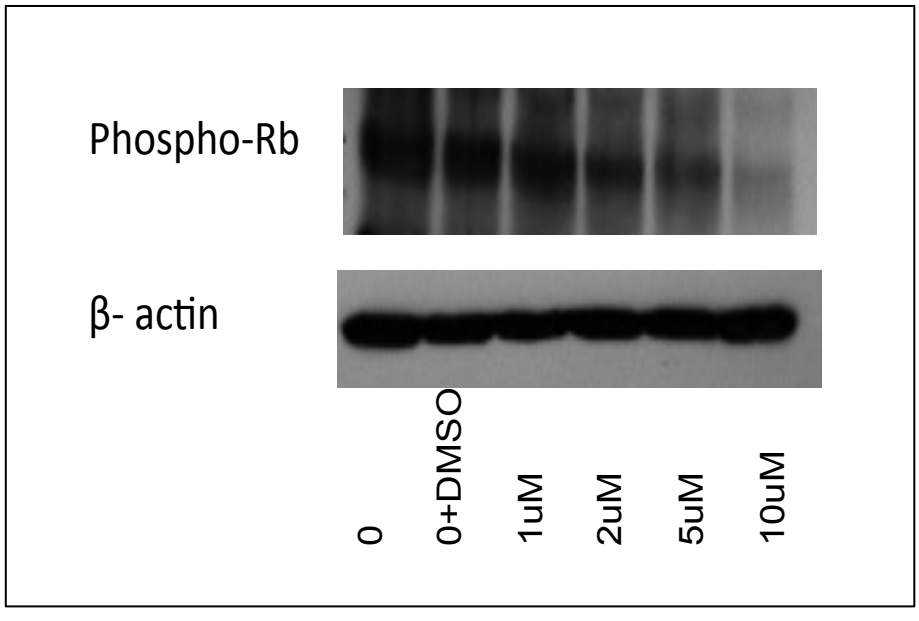


Figure 4.29 A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT7519 (1μM- 10μM) media only or DMSO control for 24 hours. The blot was probed with Phospho-Rb antibody.

CONCLUSION

Retinoblastoma protein phosphorylation is decreased by treatment with AT7519.

4.5.1.3 pp1-α/Phospho pp1-α protein expression

Protein phosphatase type 1 (pp1) is one of the major cellular serine/threonine protein phosphatases and has a crucial role in cell cycle progression. The activity of Rb in turn is regulated by phosphorylated pp1-α. Its phosphorylation is controlled by CDK2. It was hypothesized that therefore phosphorylation of pp1-α would be inhibited by AT7519.

On western blot analysis pp1-α levels were unchanged however at higher AT7519 concentrations pp1-α phosphorylation was inhibited (figure 4.30 and 4.31).

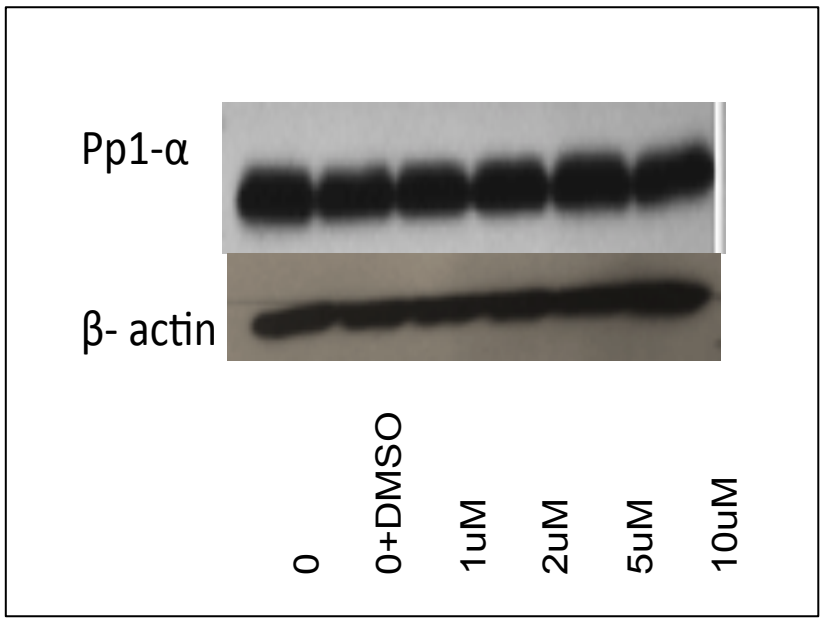


Figure 4.30 A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT7519 (1μM- 10μM) media only or DMSO control for 24 hours. The blot was probed with pp1-α antibody.

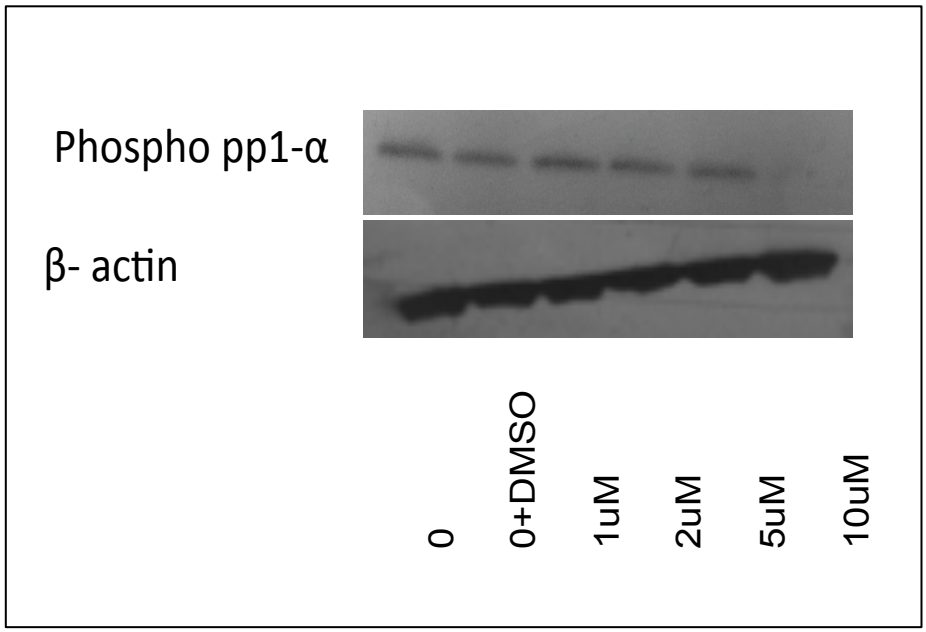


Figure 4.31 A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT7519 (1μM- 10μM) media only or DMSO control for 24 hours. The blot was probed with Phospho pp1-α antibody.

CONCLUSION

The phosphorylation of pp1-α was inhibited by AT7519 in line with its expected actions on this protein.

4.5.1.4 NPM/Phospho NPM protein expression

Nucleophosim (NPM) plays important roles in the regulation of cell proliferation and apoptosis and has been found abundant in tumour cells. The phosphorylation of NPM is facilitated by cyclin E/CDK2 which is a precursor step to centrosome duplication.

Western blot analysis was performed with NPM and phosphor NPM antibodies using cells that had been treated with AT7519 or gemcitabine (experiements performed by Owain Jones). Results showed whilst NPM levels were unchanged by either AT7519 or gemcitabine the phosphorylation of NPM was reduced by both agents (figure 4.32).

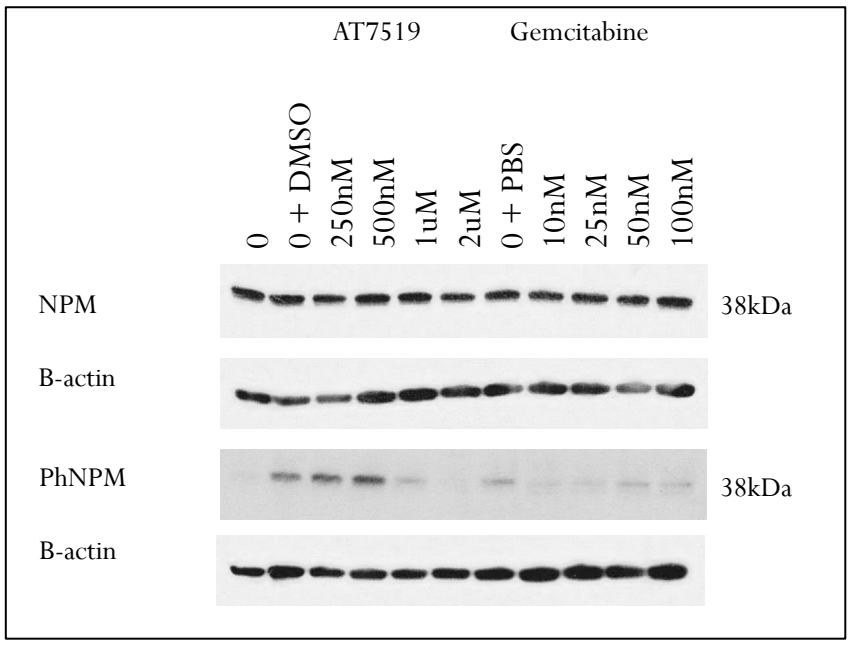


Figure 4.32 A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT7519 (250nM-2µM) or gemcitabine (10nM – 100nM) media only or DMSO control for 24 hours. The blots were probed with NPM and Phospho NPM antibody.

Further investigation was performed on cells treated with AT7519 and gemcitabine in combination. Again the levels of NPM were unchanged. When AT7519 and gemcitabine were given in combination the levels of phosphor-NPM were markedly reduced, when cells were treated with gemcitabine followed by AT7519 the effects observed were similar to that of AT7519 alone (figure 4.33).

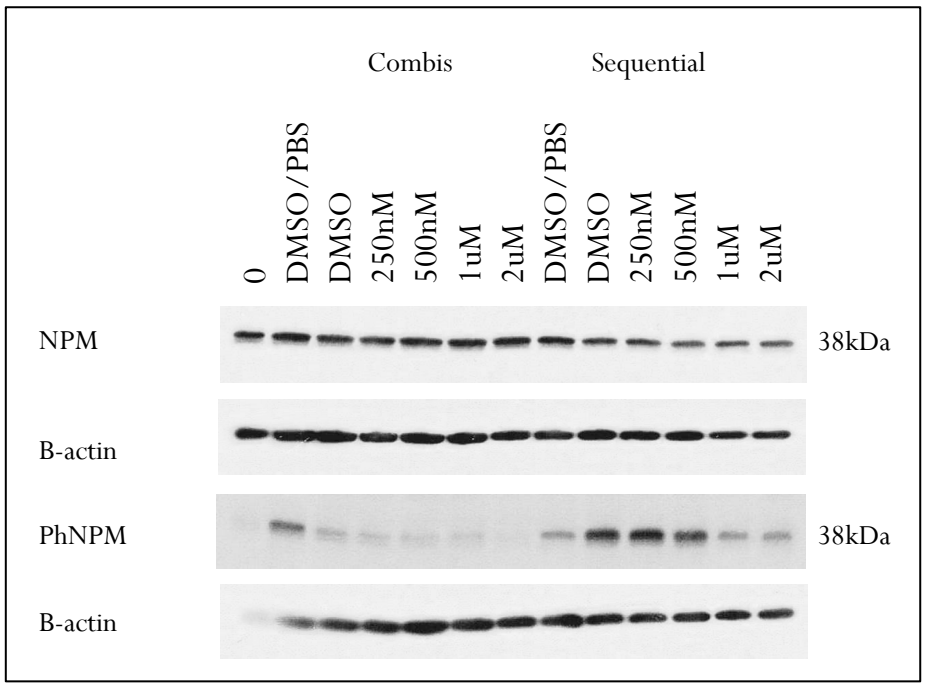


Figure 4.33 Figure showing combination and sequential addition treatment- A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT7519 (250nM – 2µM) and Gemcitabine (25nM) for 24 hours. In the sequential addition experiment gemcitabine was given for 24 then AT7519 for 24 hours. The blots were probed with NPM and PhNPM antibody.

CONCLUSION

Phosphorylation of NPM was reduced in cells treated with AT7519 and this effect was still observed when cells were treated with both AT7519 and gemcitabine but more markedly when both agents were used simultaneously.

4.5.1.5 Summary of AT7519 treatment effects

Table 4.5 summarises the effects seen on the protein expression of those tested with AT7519 or the combination of AT7519 and gemcitabine.

Protein marker	Effect on protein expression with AT7519	Effect on protein expression with AT7519 + Gemcitabine
Cdc2	↔	↔
Rb	↔	No data
Phospho Rb	↓	No data
pp1-α	↔	No data
Phospho pp1-α	↓	No data
NPM	↔	↔
Phospho NPM	↓	↓

Table 4.5 A summary table showing the protein expression effects of treatment with AT7519 or combination treatment with AT7519 and gemcitabine, (↓represents downregualtion ↔ represents no change in protein expression).

4.5.2 Western blotting of downstream targets AT13387

Western blotting was performed in order to establish the downstream targets/effects of treatment with AT13387. A number of potential targets were explored both to demonstrate activity and establish any potential biomarkers for AT13387.

Inhibition of Hsp90 has been shown to affect a number of oncogenes and proteins important for the growth and survival of cancer cells. This includes molecules involved in cell survival, growth and cell cycle progression. We examined the effect of AT13387 inhibition on a number of these proteins including those involved in the MAP Kinase and PI3K pathways. A summary of the effects on protein expression are summarized in table 4.6 at then end of this section. Experiments in this section were performed by Elisabeth Shaw.

4.5.2.1 Akt/Phospho Akt protein expression

Akt is a downstream effector of phosphoinositide 3-kinase and mediates biological actions in respect to anti-apoptotic responses. HSP90 binding to Akt reduces sensitivity of cells to apoptosis inducing stimuli therefore it was hypothesized that AT13387 may play a role in regulating Akt and its phosphorylation.

Miapaca-2 cells were treated with AT13387 for 18 hours. This resulted in significantly decreased levels of phosphor Akt and decreased levels of Akt with increasing concentrations of AT13387 (figure 4.34).

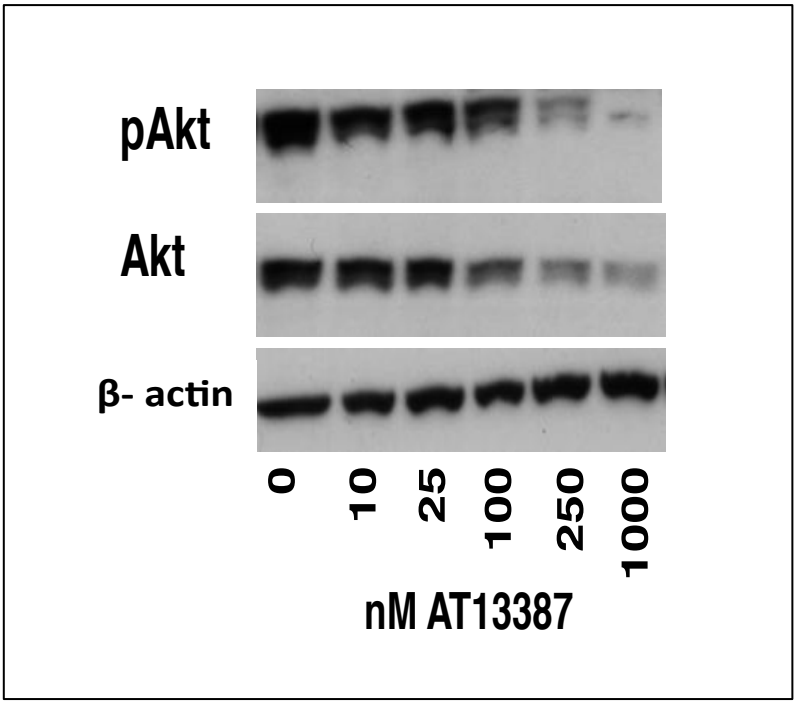


Figure 4.34 A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT13387 (10nM- 1000nM) or media control for 18 hours. The blots were probed with Akt and phospho Akt antibody.

When Miapaca-2 cells were treated with AT13387 plus gemcitabine it was noted that decreased concentrations of Akt were observed both when the agents were given together (figure 4.35) and when agents were added sequentially, the effect was less marked when agents added sequentially (figure 4.36).

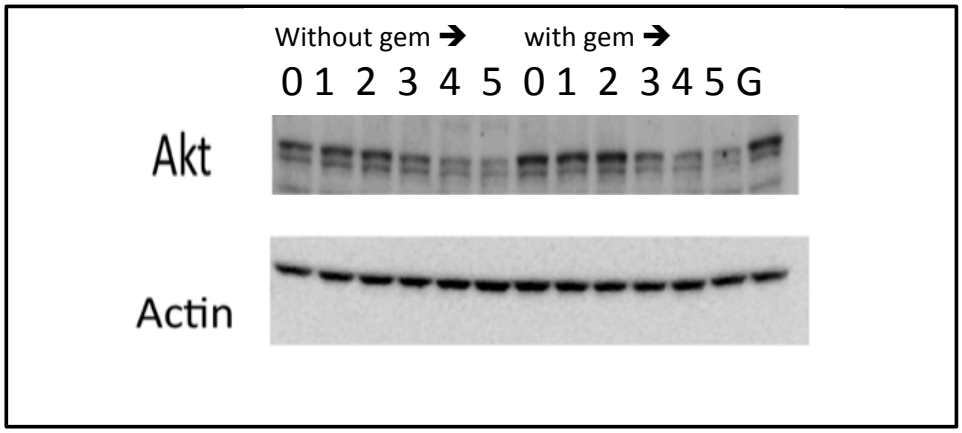


Figure 4.35 Figure showing combination treatment – A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT13387 (1. 10nM, 2. 25nM, 3. 100nM, 4. 250nM, 5. 1000nM) or AT13387 with Gemcitabine (500nM) for 18 hours. G represents treatment with gemcitabine 500nM alone. The blots were probed with Akt antibody.

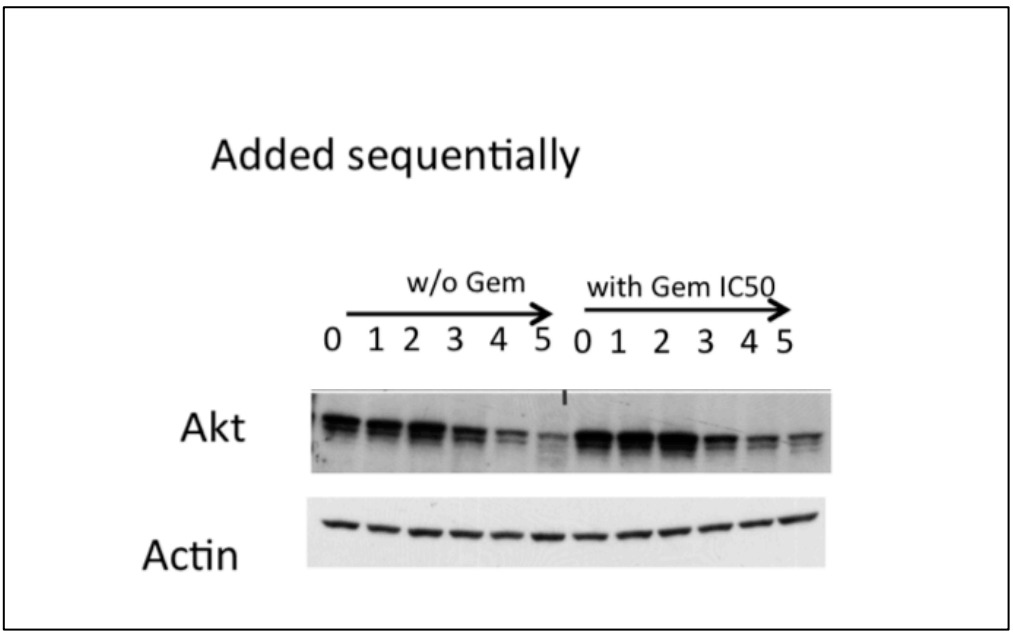


Figure 4.36 A figure showing the sequential addition of agents - A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT13387 (1. 10nM, 2. 25nM, 3. 100nM, 4. 250nM, 5. 1000nM) or media control as a single agent or AT13387 with Gemcitabine (500nM) for 18 hours. For combination study cells were treated with gemcitabine for 24 hrs then AT13387 was added for 24hrs. The blots were probed with Akt antibody.

CONCLUSION

Treatment of Miapaca-2 cells with AT13387 resulted in both decreased levels of Akt and Phospho Akt. When cells were treated with both AT13387 and Gemcitabine Akt levels decreased irrespective of drug scheduling but was less marked when gemcitabine was given prior to AT13387.

4.5.2.2 S6/Phospho S6 protein expression

Phosphorylation of the S6 ribosomal protein correlates with increases mRNA transcripts that encode for proteins involved in cell cycle progression and factors necessary for translation. Decreased phosphorylation of S6 should correlate with HSP90 inhibition.

Miapaca-2 cells treated with AT13387 showed significant decreases of phosph-S6 at 18 hours after treatment, levels of S6 were unchanged (figure 4.37).

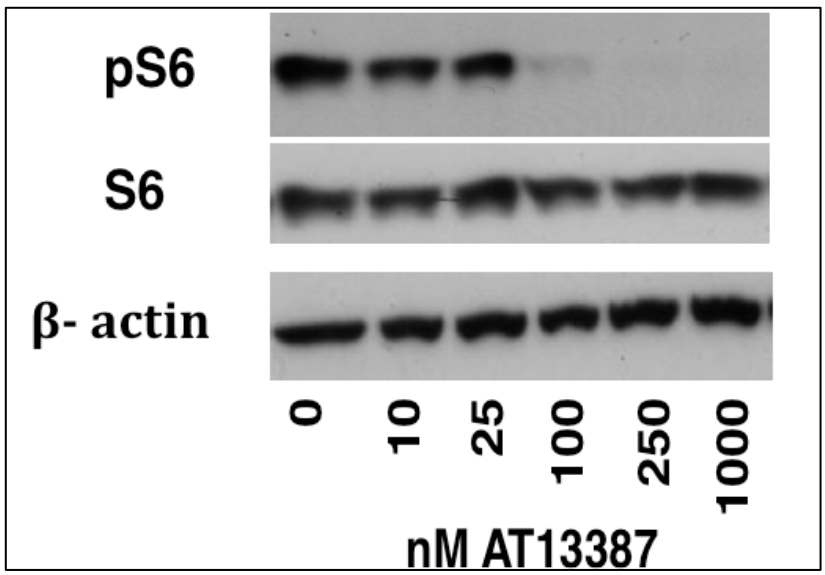


Figure 4.37 A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT13387 (10nM- 1000nM) or media control for 18 hours. The blots were probed with S6 and phospho S6 antibody.

When cells were treated with AT13387 and gemcitabine concurrently the decreased phosphor S6 was also observed and the effect was more marked at lower doses of AT13387 (figure 4.38). This was also true of sequential scheduling of gemcitabine followed by AT13387 (figure 4.39).

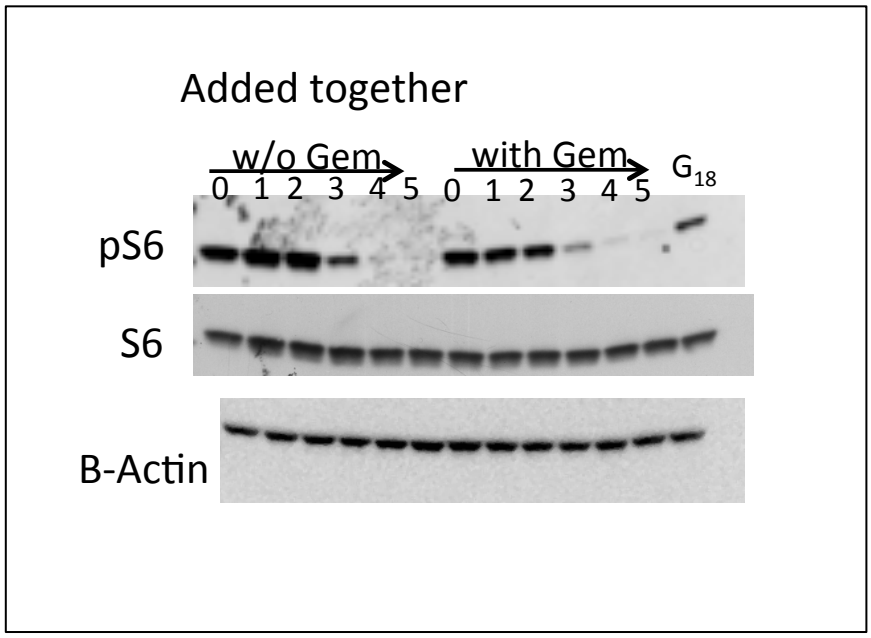


Figure 4.38 A figure showing combination treatment with agents - A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT13387 (1. 10nM, 2. 25nM, 3. 100nM, 4. 250nM, 5. 1000nM) or media control as a single agent or AT13387 with gemcitabine (500nM) for 18 hours. G_{18} represents treatment with gemcitabine 500nM alone. The blots were probed with S6 and phospho S6 antibody.

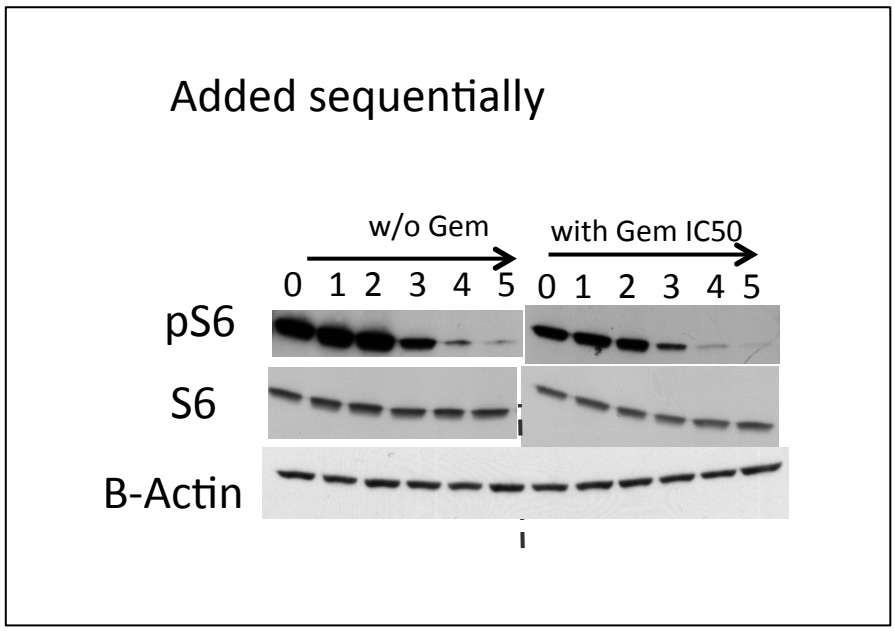


Figure 4.39 A figure showing the sequential addition of agents – A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT13387 (1. 10nM, 2. 25nM, 3. 100nM, 4. 250nM, 5. 1000nM) or media control as a single agent or AT13387 with gemcitabine (500nM) for 18 hours. For combination study cells were treated with gemcitabine for 24 hrs then AT13387 was added for 24hrs. The blots were probed with S6 and phospho S6 antibody.

CONCLUSION

Treatment with AT13387 resulted in decreased phosphorylation of S6, this was also noted when AT13387 was combined with gemcitabine regardless of dosing schedule and the effect was noted at lower doses of AT13387.

4.5.2.3 Raf-1 protein expression

HSP90 is essential for raf-1 protein stability and it localization in the cell. Raf-1 activation initiates the MAPK (mitogen-activated protein kinase) cascade that has critical regulatory functions in determining cell proliferation, differentiation, apoptosis and oncogenic transformation. It was hypothesized that AT13387 treatment would result in decreased levels of Raf-1.

When Miapaca-2 cells were treated with increasing concentrations decreased levels of Raf-1 were noted (figure 4.40).

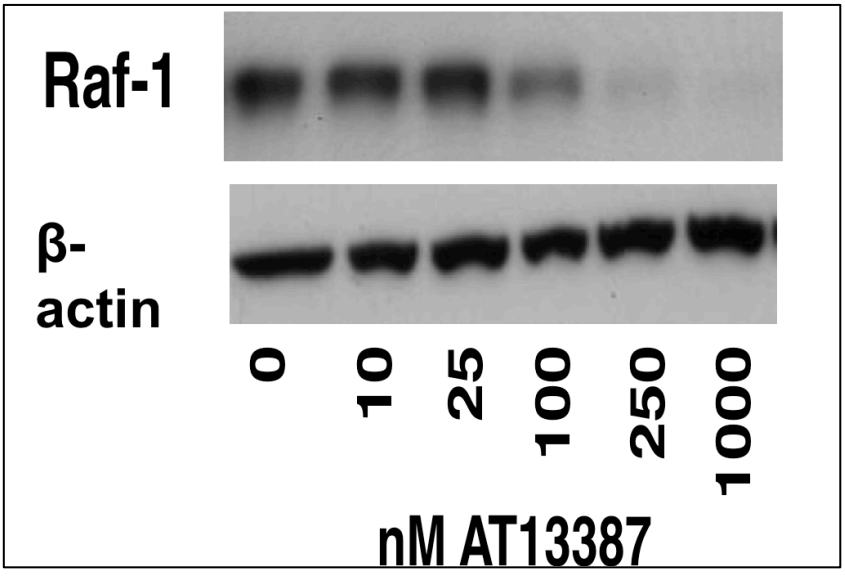


Figure 4.40 A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT13387 (10nM- 1000nM) or media control for 18 hours. The blot was probed with Raf-1 antibody.

The combination of gemcitabine and AT13387 showed similar effects to that of AT13387 alone (4.41). When the agents were given sequentially (gemcitabine for 24 hours followed by AT13387) the effects on decreasing protein expression were more marked and observed at lower doses of AT13387 (figure 4.42). This supports the notion that gemcitabine as a nucleoside analogue acts mostly on dividing and replicating cells.

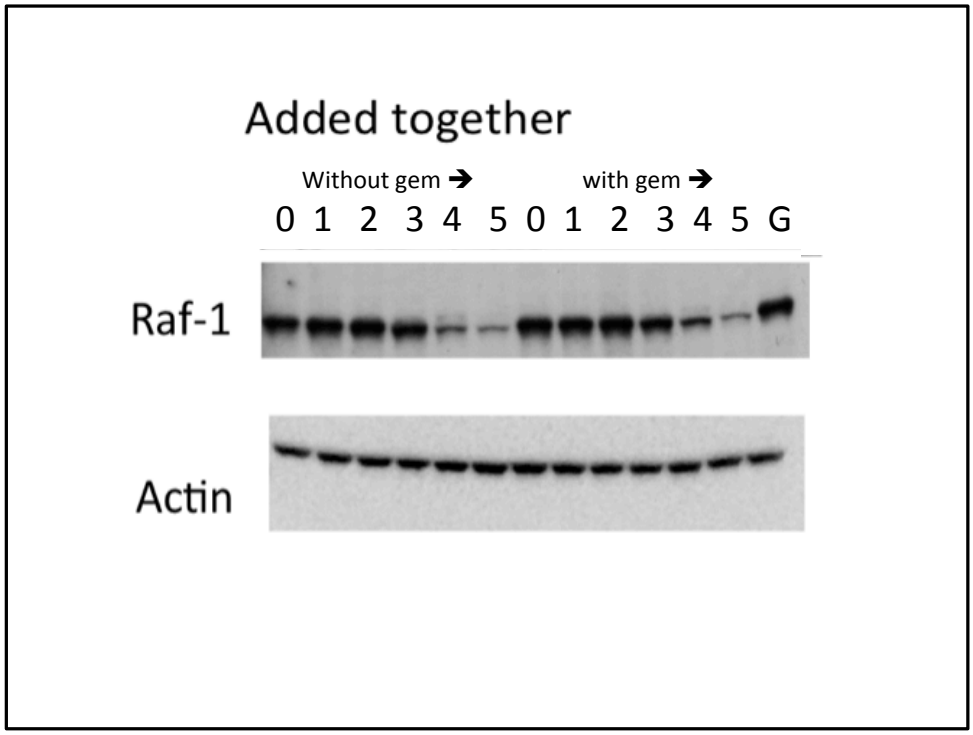


Figure 4.41 A figure showing combination treatment of agents - A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT13387 (1. 10nM, 2. 25nM, 3. 100nM, 4. 250nM, 5. 1000nM) or media control as a single agent or AT13387 with gemcitabine (500nM) for 18 hours. G represents treatment with gemcitabine 500nM alone. The blots were probed with Raf-1 antibody.

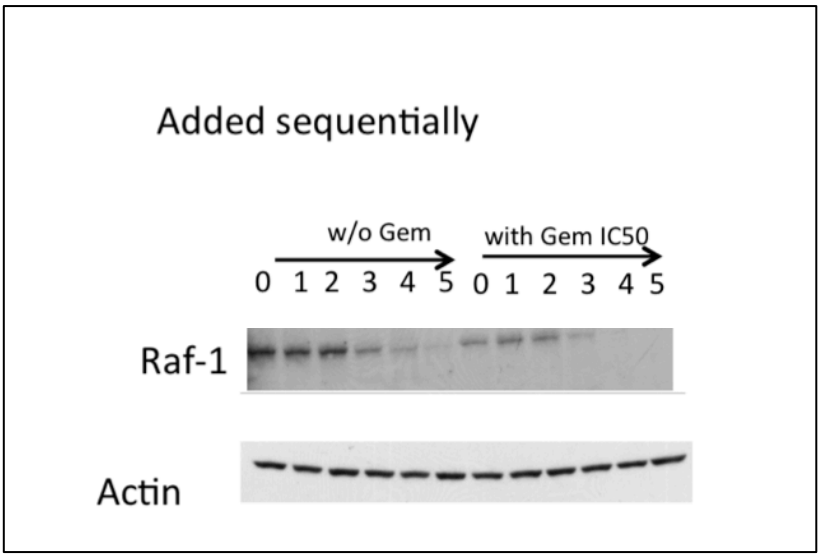


Figure 4.42 A figure showing the sequential addition of agents – A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT13387 (1. 10nM, 2. 25nM, 3. 100nM, 4. 250nM, 5. 1000nM) or media control as a single agent or AT13387 with gemcitabine (500nM) for 18 hours. For combination study cells were treated with gemcitabine for 24 hrs then AT13387 was added for 24hrs. The blots were probed with Raf-1 antibody.

CONCLUSION

In line with its expected actions AT13387 reduced the protein expression levels of Raf-1, the effect on protein expression was more marked when cells were treated sequentially with gemcitabine followed by AT13387 and observed at lower doses of AT13387.

4.5.2.4 CDK4 protein expression

CDK4 protein is a member of the cyclin dependent kinase family of enzymes that has been found to be associated with tumourgenesis in a variety of cancers and forms a complex with HSP90 and cdc37.

When Miapaca-2 cells were treated with AT13387 significant decreases in CDK4 protein expression were noted (figure 4.43).

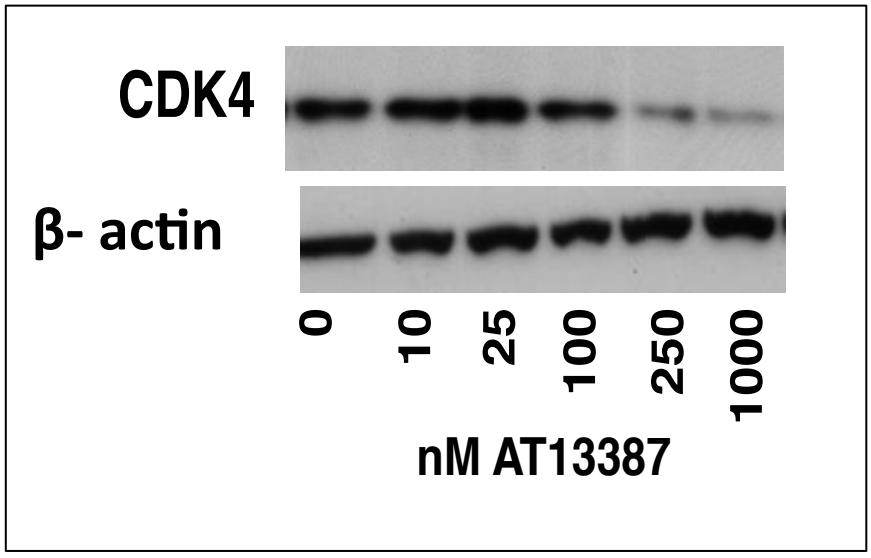


Figure 4.43 A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT13387 (10nM- 1000nM) or media control for 18 hours. The blots were probed with CDK4 antibody

The combination of AT13387 and gemcitabine showed similar effects to that of AT13387 alone (figure 4.44). Again when gemcitabine and AT13387 were given sequentially the same decreased protein expression was observed but this was noted at decreased concentrations of AT13387 (figure 4.45).

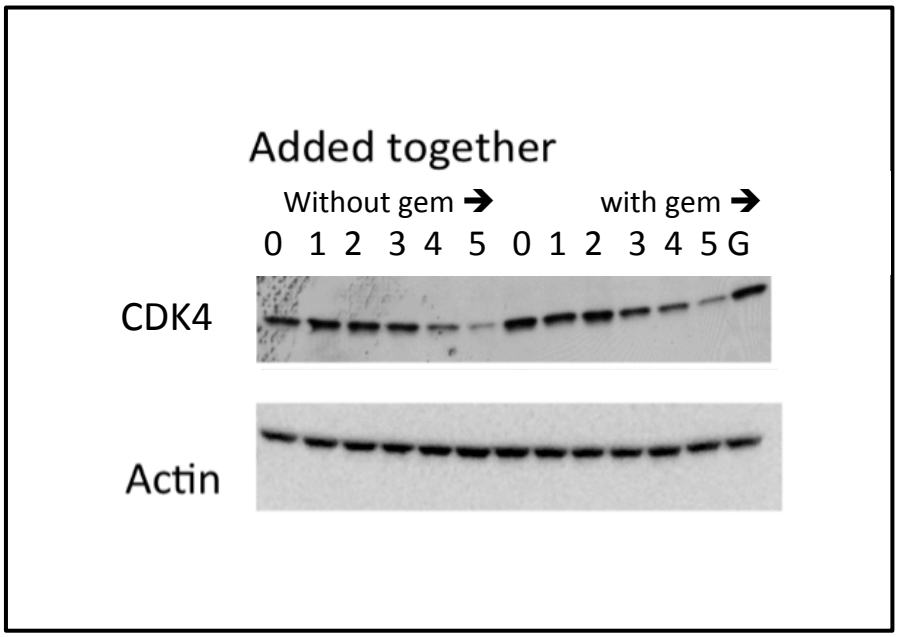


Figure 4.44 A figure showing the combination treatment of agents – A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT13387 (1. 10nM, 2. 25nM, 3. 100nM, 4. 250nM, 5. 1000nM) or media control as a single agent or AT13387 with gemcitabine (500nM) for 18 hours. G represents treatment with gemcitabine 500nM alone. The blots were probed with CDK4 antibody.

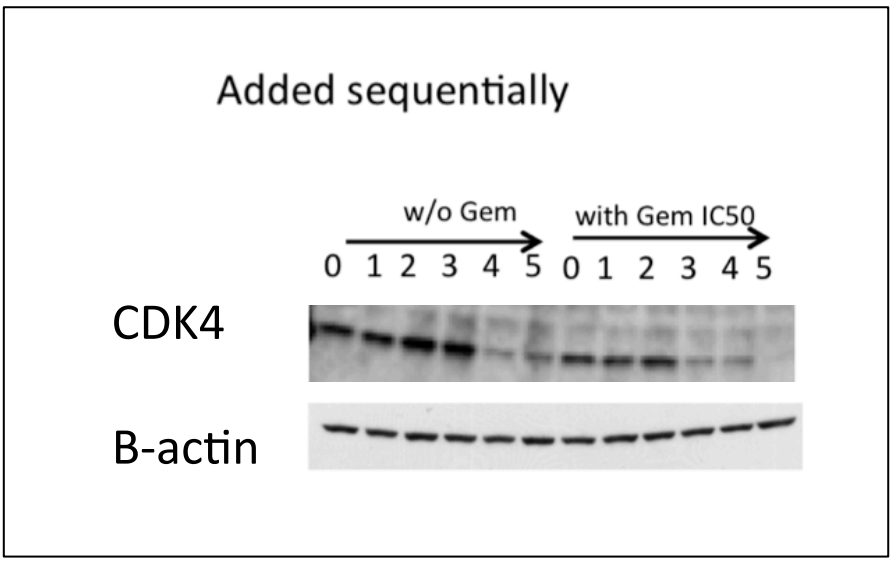


Figure 4.45 A figure showing the sequential addition of agents – A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT13387 (1. 10nM, 2. 25nM, 3. 100nM, 4. 250nM, 5. 1000nM) or media control as a single agent or AT13387 with Gemcitabine (500nM) for 18 hours. For combination study cells were treated with gemcitabine for 24 hrs then AT13387 was added for 24hrs. The blot was probed with CDK4 antibody.

CONCLUSION

AT13387 reduced the protein expression levels of CDK4, the effect on protein expression was more marked when cells were treated sequentially with gemcitabine followed by AT13387 and observed at lower doses of AT13387.

4.5.2.5 HSP70 protein expression

HSP70 is a heat shock protein that as a molecular chaperone accumulates in cells at times of cellular stresses, including oncogenic transformation. HSP70 is a co chaperone to HSP90 inhibition.

A significant increase in HSP70 was observed following treatment with AT13387 (figure 4.46).

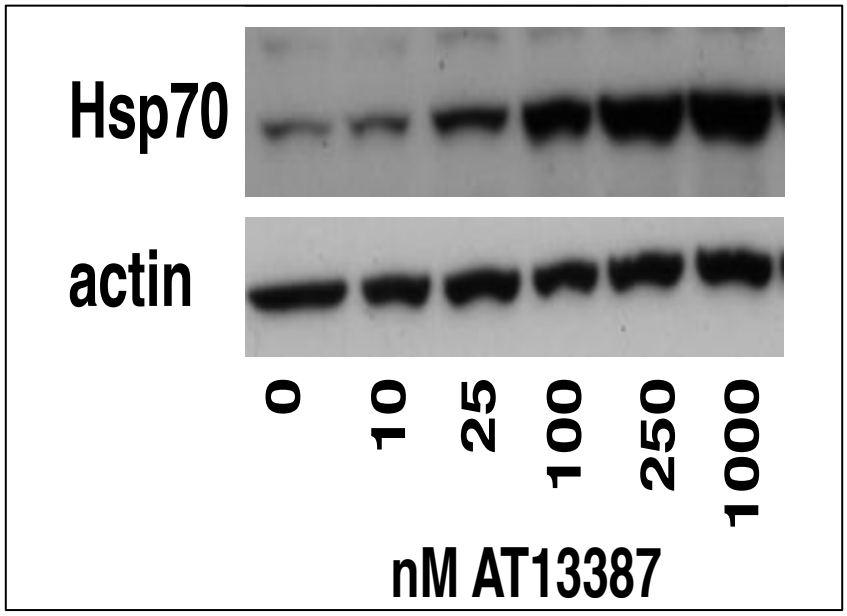


Figure 4.46 A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT13387 (10nM- 1000nM) or media control for 18 hours which was probed with HSP70 antibody.

When Miapaca-2 cells were treated with AT13387 plus gemcitabine the same effects were observed. There was no noted difference between combination therapy and sequential addition of agents (figure 4.47 and 4.48).

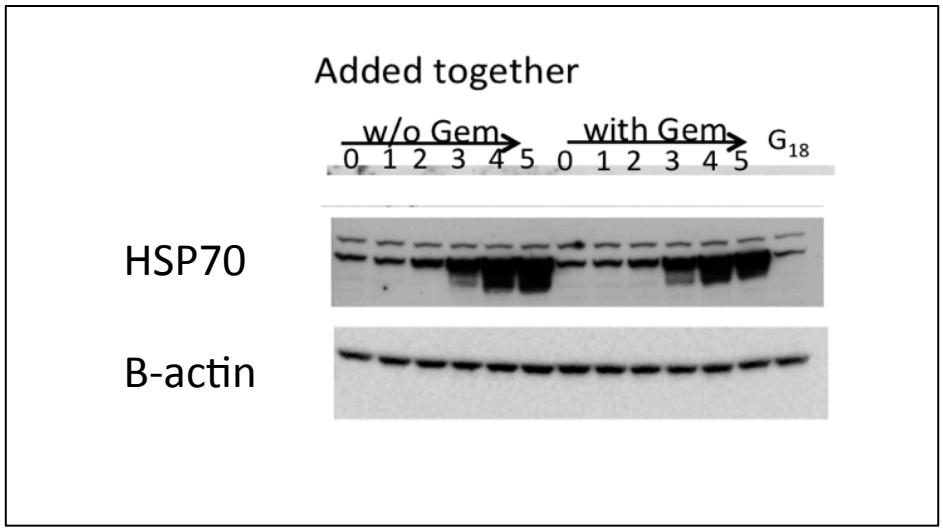


Figure 4.47 A figure showing the combination experiments- A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT13387 (1. 10nM, 2. 25nM, 3. 100nM, 4. 250nM, 5. 1000nM) or media control as a single agent or AT13387 with gemcitabine (500nM) for 18 hours. G_{18} represents treatment with gemcitabine 500nM alone. The blots were probed with HSP70 antibody.

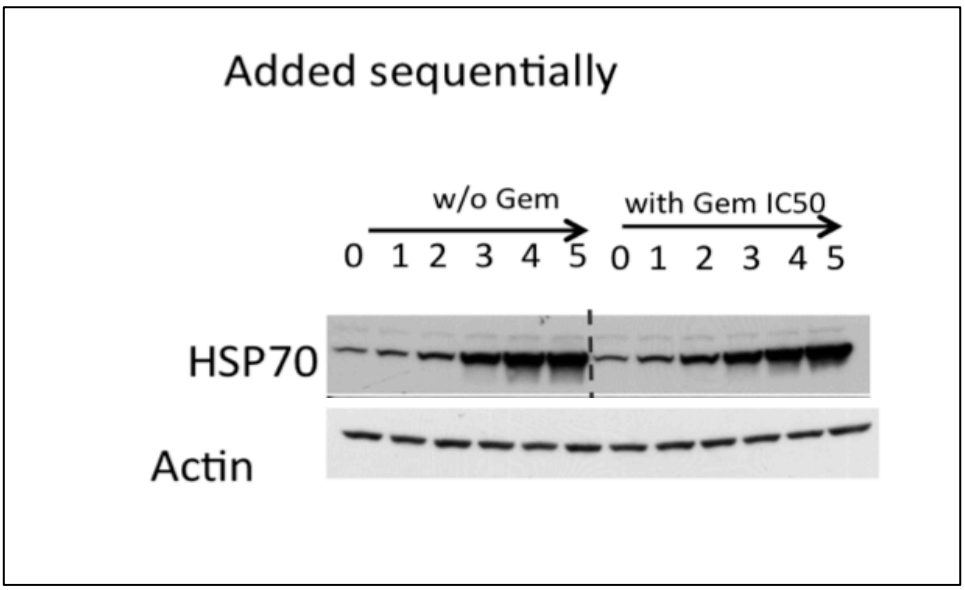


Figure 4.48 A figure showing the sequential addition of agents – A western blot assay of protein extracts from pancreatic cancer cell line Miapaca-2 treated with AT13387 (1. 10nM, 2. 25nM, 3. 100nM, 4. 250nM, 5. 1000nM) or media control as a single agent or AT13387 with gemcitabine (500nM) for 18 hours. For combination study cells were treated with gemcitabine for 24 hrs then AT13387 was added for 24hrs. The blots were probed with HSP70 antibody.

CONCLUSION

Cells treated with AT13387 showed upregulation of HSP70 protein compared to control, the same effect of upregulation was observed in combination with gemcitabine regardless of drug scheduling.

4.5.2.6 Summary of AT13387 effects

The table below summarises the effects seen on the protein expression of those tested with AT13387 or the combination of AT13387 and gemcitabine.

Protein Marker	Effect on protein expression with AT13387	Effect on protein expression with AT13387 + Gemcitabine
Akt	↓	↓
Phospho Akt	↓	↓
S6	↔	↔
Phospho S6	↓	↓↓
Raf-1	↓	↓↓
CDK4	↓	↓↓
HSP70	↑	↑

Table 4.6 A summary table showing the protein expression effects of treatment with AT13387 or combination treatment with AT13387 and gemcitabine (↓ represents downregulation, ↑ represents upregulation, ↔ represents no change in protein expression).

CHAPTER 5: RESULTS *IN VIVO*

In vivo studies with agents AT7519, AT13387, gemcitabine and matched treatment controls were undertaken using 6-8 week old BALB/c-Nude mice. Experiments examined tumour growth, tolerability and efficacy of treatment.

5.1 TUMOUR GROWTH EXPERIMENTS

Initial experiments were performed to establish the optimal cell line to take forward into the xenograft model. Cell line characteristics deemed desirable included those which would generate a measurable tumour that would grow over an acceptable time frame in order to allow observations and detect any differences between control groups and treatment groups.

The cell lines tested were SUIT-2 and Miapaca-2 from stocks frozen down as per the methodology section 3.1.2**.** Four mice per group were inoculated with subcutaneous tumours to each shoulder (see table 5.1 and 5.2).

Mice were injected on day 0 and observed for up to 28 days. Tumour measurements were performed by a single observer using calipers measuring

length, width and height of tumours, to establish tumour volume using the equation:

(length x width x height) x 0.5236 = tumour volume

Mice were also weighed daily and general observations on behavior were made.

Both cell lines produced palpable and measurable tumours that increased in size in an exponential manner (see figure 5.1, 5.2 and 5.3). Experiments were terminated when experimental end points were reached: tumours exceeding 17mm diameter, sustained weight loss (72 hrs.) of >20%, displayed adverse behaviour/physical condition or the planned number of days reached relevant to the experiment (detailed in section 3.2).

Of the 4 SUIT-2 mice, 2 were sacrificed at day 19 owing to tumour volume and tumour ulceration, one was sacrificed on day 23 owing to tumour volume and the last sacrificed at the experimental endpoint of day 28, the tumour was noted to be ulcerated at this point.

Of the 4 Miapaca-2 mice three were sacrificed on day 23 owing to tumour volumes and one reached 28 days.

Subject	Right shoulder	Left shoulder	Notes
Mouse 1	200µl injection	200µl injection	Hematoma from right injection
Mouse 2	200µl injection	200µl injection	
Mouse 3	200µl injection	200µl injection	Small loss left
Mouse 4	200µl injection	200µl injection	Small loss left

Table 5.1 A table showing the injections of Miapaca-2 tumours of 4 mice of 200µl per tumour. The site of injections, volumes and any notes/complications shown.

Subject	Right shoulder	Left shoulder	Notes
Mouse 1	200µl injection	200µl injection	
Mouse 2	200µl injection	200µl injection	Small loss both sides
Mouse 3	200µl injection	175µl injection	Bleed on injection to left, last 15µl not injected
Mouse 4	150µl injection	150µl injection	Not enough tumour suspension left therefore 150µl both sides

Table 5.2 A table showing the injections of SUIT-2 tumours of 4 mice of 200µl per tumour. The site of injections, volumes and any notes/complications shown.

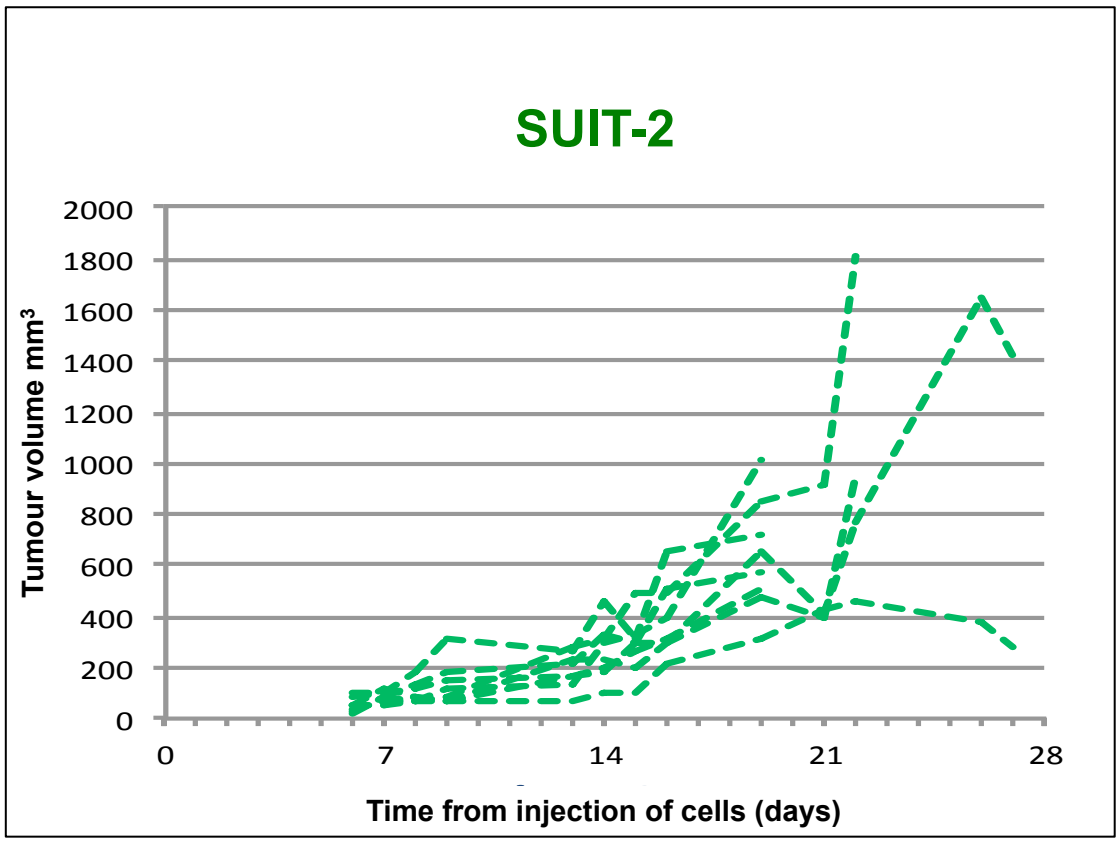


Figure 5.1 A graph showing the individual tumour growth of 8 tumours (four mice, 2 tumours per mouse) generated from SUIT-2 tumour matrix. The x-axis shows time from injection of cells in days, the y-axis shows the tumour volumes in mm^3. Each green dashed line represents an individual tumour. The graph shows tumours becoming measurable at day 6 and growth of tumours, the majority in an exponential manner.

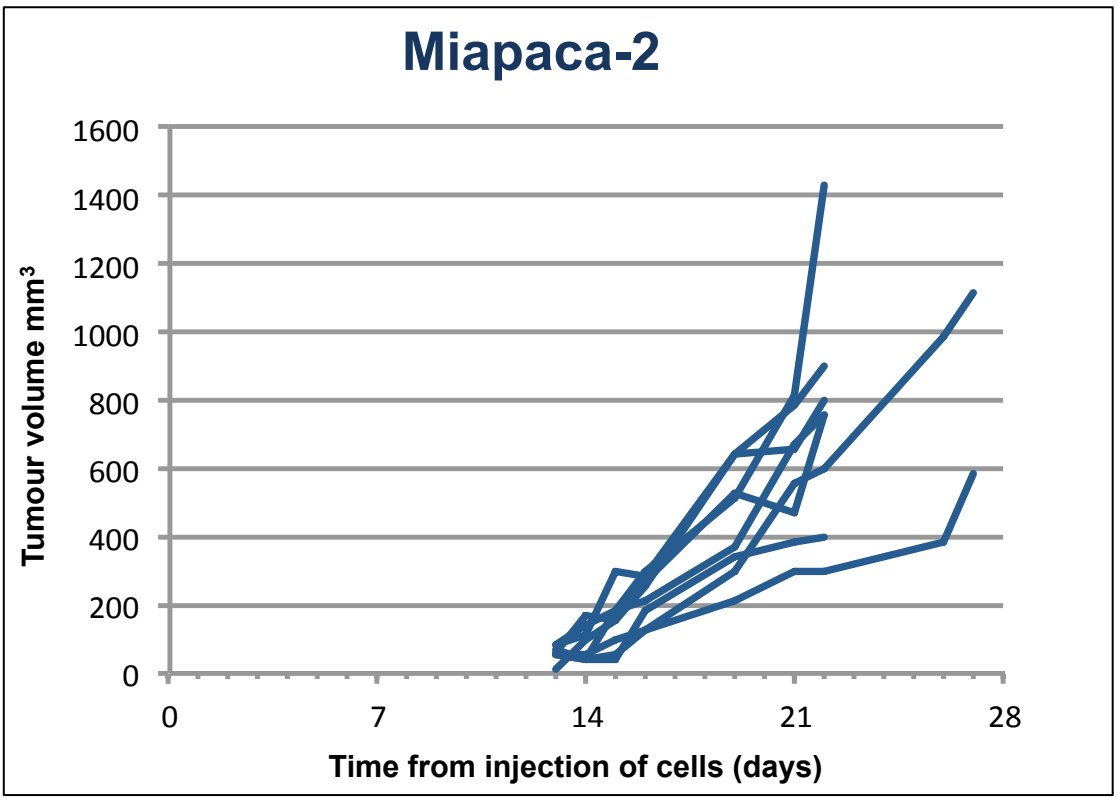


Figure 5.2 A graph showing the individual tumour growth of 8 tumours (four mice, 2 tumours per mouse) generated from Miapaca-2 tumour matrix. The x-axis shows time from injection of cells in days, the y-axis shows the tumour volumes in mm^3. Each green dashed line represents an individual tumour. The graph shows tumours becoming measurable at day 13 and growth of tumours in an exponential manner.

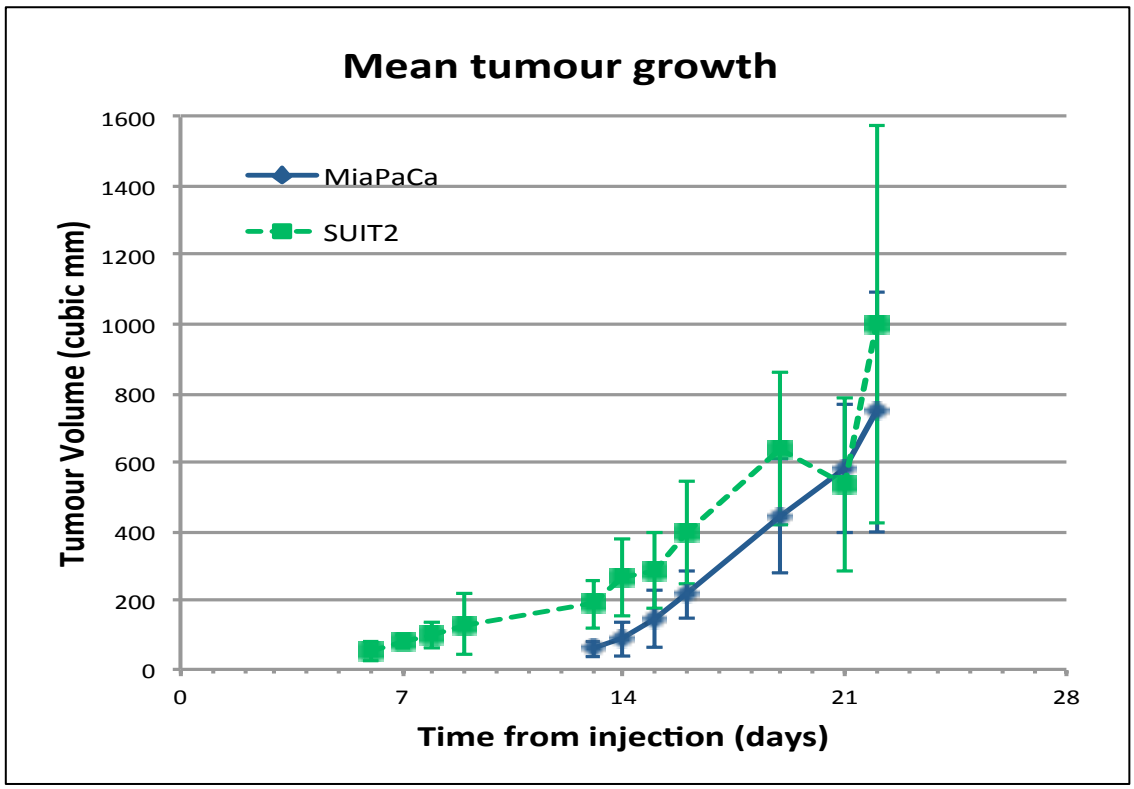


Figure 5.3 A graph showing the mean tumour growth of all tumours generated from Miapaca-2 and SUIT-2 tumour matrix injections. The x-axis shows time from injection of cells in days, the y-axis shows tumour volume in mm^3. The graph shows exponential tumour growth demonstrated by both tumour cell lines and SUIT-2 cells becoming measurable around day 6 whilst Miapaca-2 tumours became measurable at day 13.

Post sacrifice tumours were harvested and examined by a pathologist (Dr F Campbell) to examine tumour characterisitics.

SUIT-2 tumours grew in a fashion generating more rounded tumours with a greater height compared to miapaca tumours. This allowed earlier detection and mesurements to be performed at 6-7 days post injection. At the later stages of tumour growth tumours became more fluctuant centrally with collapse that

effected tumour measurements. In the SUIT-2 group 2 animals needed to be sacrificed owing to this phenomenon leading to tumour ulceration. On dissection there was a central exudate (ranging from pus like to watery), tumours were centrally hollow with largely peripheral vascularisation. Histologically necrosis was observed (see figure 5.4, slide A).

Miapaca-2 tumours grew in a flatter more nodular manner compared to SUIT-2 tumours. Each tumour was composed of multiple smaller growths that were firmily adhered to each other as shown in figure 5.4, slide B. Some nodules grew at a small distance from the central tumours. After dissection less necrosis was observed compared to SUIT-2 tumours with an absence of central necrosis.

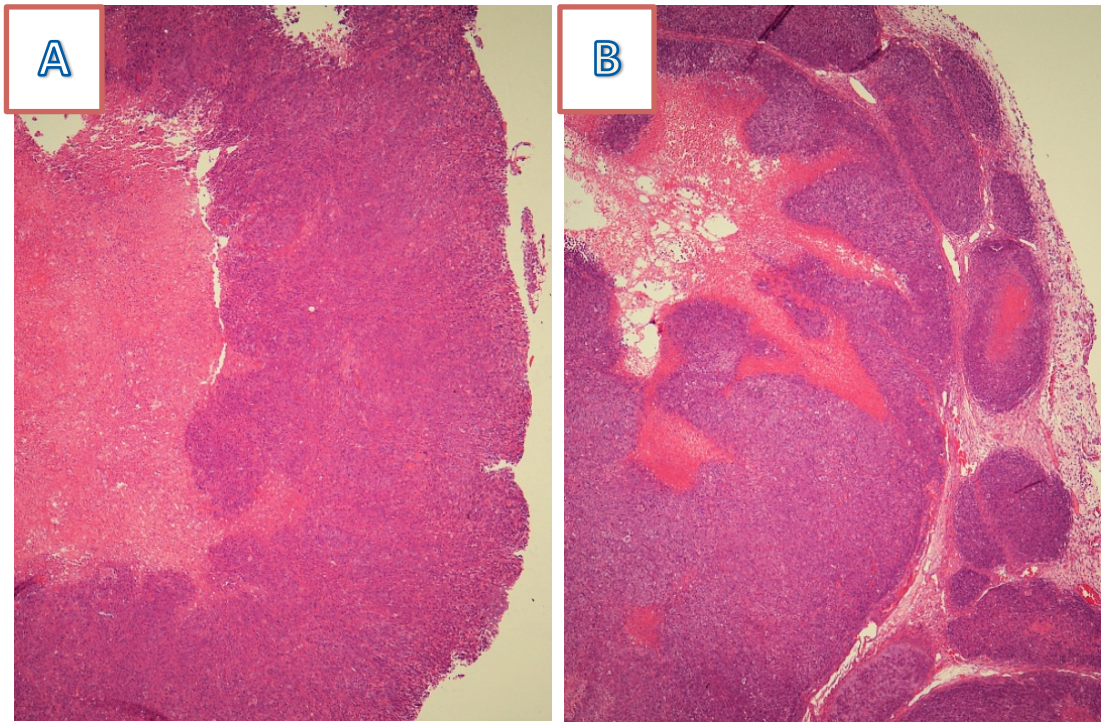


Figure 5.4 Photographs of histopathology slides of the tumours generated.

A) A SUIT-2 tumour showing central tumour necrosis .

B) A Miapaca-2 tumour showing tumours consisting of multiple nodules.

CONCLUSION

After taking into account the characteristics of each cell line, further experiments in the xenograft model were performed using the Miapaca-2 cell line. This was owing to the more uniform growth observed without necrosis and subsequent depression of tumours that could effect tumour growth calculations in future experiments. The characteristic observed in the SUIT-2 tumours of developing ulceration in half of the subjects was also deemed undesirable in future experiments.

5.2 VALIDATION OF TUMOUR MEASUREMENT

In order to ensure validity of tumour measurements taken in subsequent studies comparison was made between measurements of tumours performed *in vivo* verses *ex vivo*.

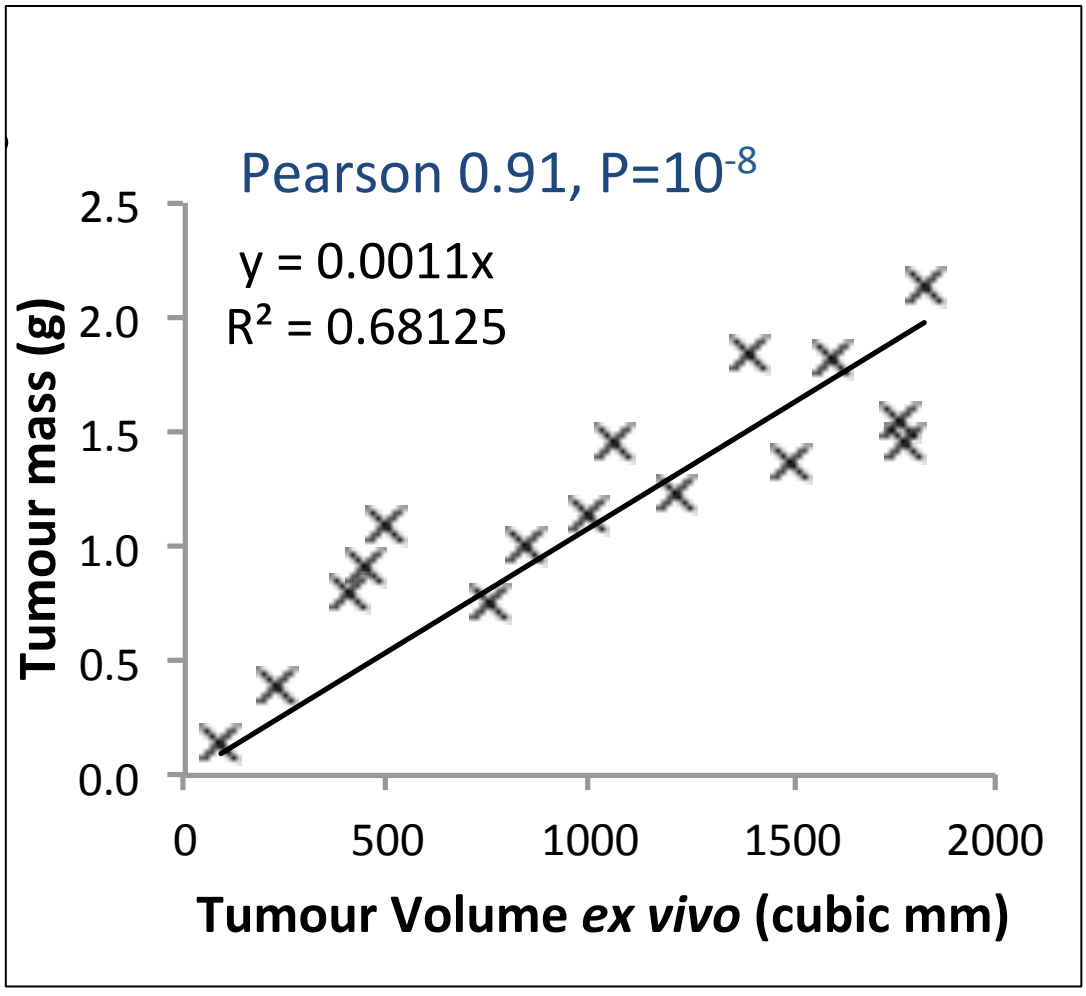


Figure 5.5 A graph showing *ex vivo* correlation of tumour volume and mass (16 tumours total, 8 mice with 2 tumours per animal). The x-axis shows tumour volume *ex vivo* in mm^3 and the y-axis shows tumour mass in grams. Pearson's correlation data shown.

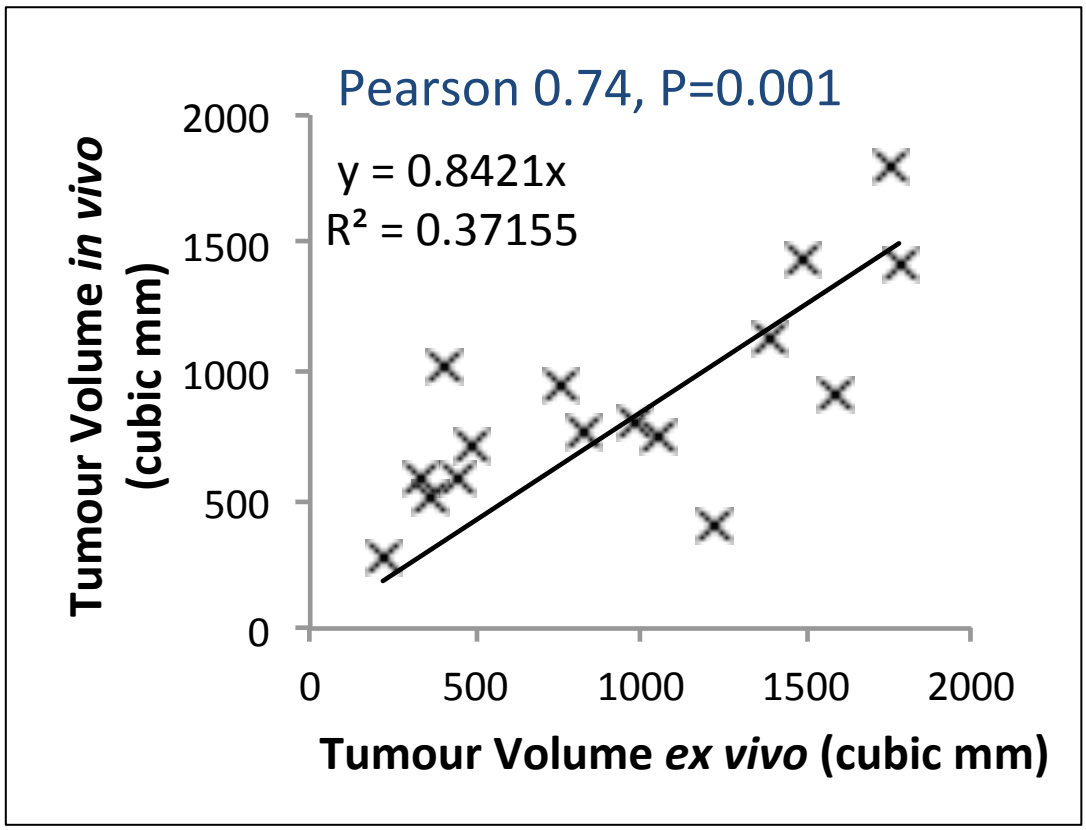


Figure 5.6 A graph showing correlation of tumour volume calculations *in vivo* and *ex vivo* (16 tumours total, 8 mice with 2 tumours per animal). The x-axis shows tumour volumes *ex vivo* in mm^3 and the y-axis shows tumour volumes *in vivo* in mm^3, Pearson's correlation data shown.

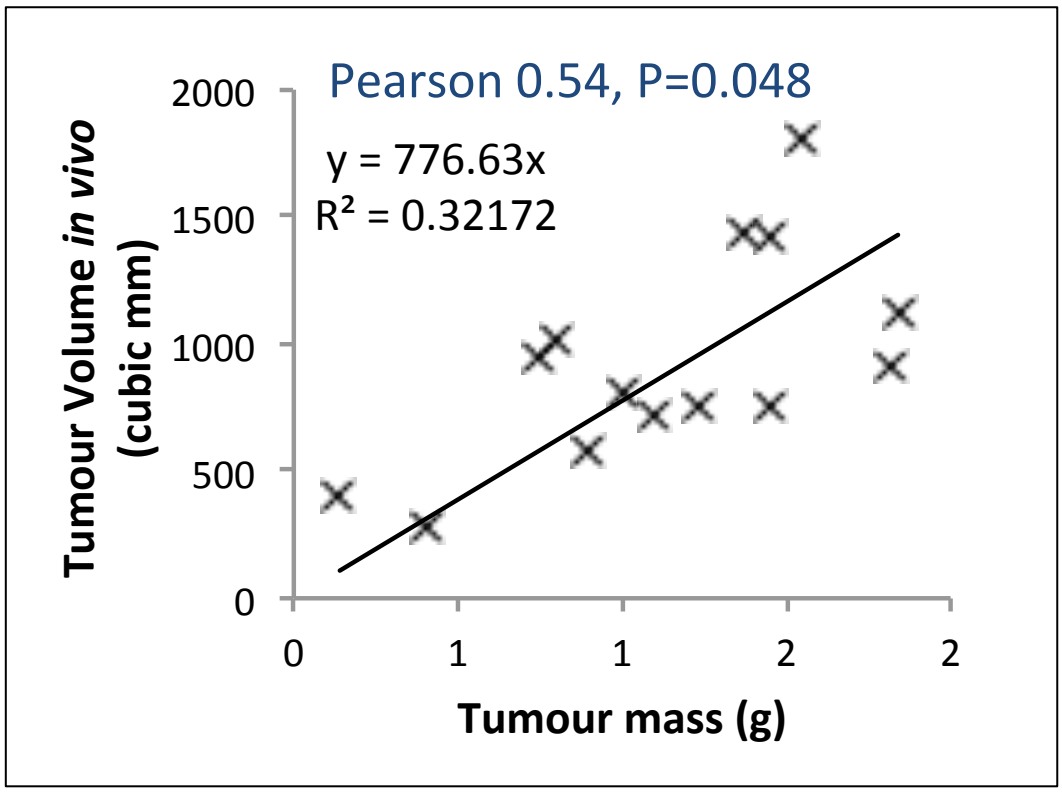


Figure 5.7 A graph showing correlation of tumour volume calculations *in vivo* with tumour mass *ex vivo* (16 tumours total, 8 mice with 2 tumours per animal). The x-axis shows tumour mass *ex vivo* in grams and the y-axis shows tumour volume *in vivo* in mm^3.

The comparative results of *in vivo* measurements with *ex vivo* measurements and weights were plotted for each tumour generated and the Pearson product-moment correlation coefficient was calculated to measure the linear correlation between the variables. The ex vivo measurements of tumour volume correlated with tumour mass ex vivo shown in figure 5.5. The results showed a positive correlation between the *in vivo* measurements and both the *ex vivo* tumour measurements and weight measurements (shown in figure 5.6 and 5.7). The correlation was not strong but none-the less a positive correlation.

CONCLUSION

Measurements confirmed that there was a positive correlation between the *in vivo* measurements performed and the *ex vivo* measurements obtained. The relationship between mass and volume, although significant, was weak. Indicating the heterogeneity of the tumours, but this does not detract from validity of the caliper measurements, which on this basis can be considered to be a justified measure of the expansion of the tumour (albeit not the only way this could be assessed). To ensure minimal variation in the results obtained a single observer performed caliper measurements throughout all experiments (AT).

5.3 SINGLE AGENT TOLERABILITY STUDIES

5.3.1 Gemcitabine single agent tolerability

The gemcitabine tolerability for this model was tested in 3 non tumour-bearing mice. The dose of gemcitabine trialed was based on previous literature experience in pancreatic xenograft models. The dose of 100mg/kg twice per week was tested for a total treatment period of 14 days. During the treatment an initial fall in weight was observed that decreased with further doses of gemcitabine (shown in figure 5.8). No animal had to be sacrificed due to this weight loss. As a single agent this was deemed the maximal tolerated dose of gemcitabine but owing to the fact that

gemcitabine would be used in combination studies the decision was made to use a decreased dose of 50mg/kg twice per week in future studies. This avoided significant weight loss that might have prevented the experimental end point being reached.

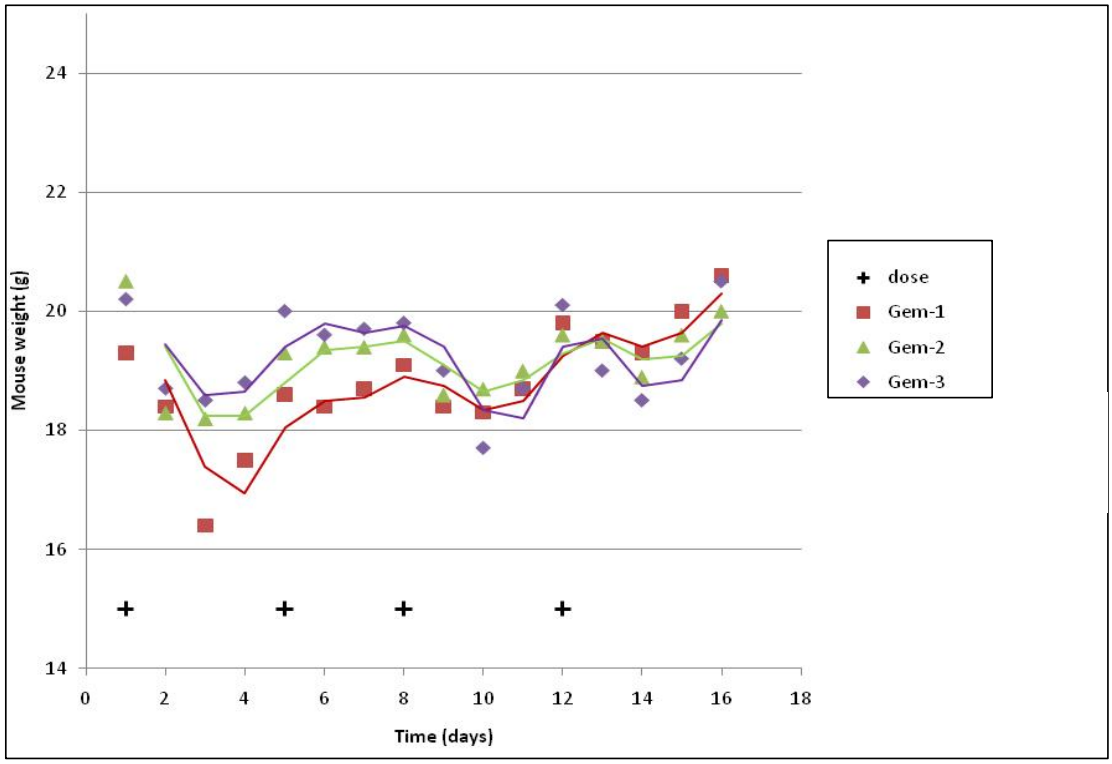


Figure 5.8 A graph showing gemcitabine tolerability: the x-axis shows time of experiment in days, the y-axis shows mouse weight in grams. The crosses represent a dose of gemcitabine. Lines of best fit were produced with a 2-point moving average. 3 mice were dosed at 100mg/kg twice per week with gemcitabine.

CONCLUSION

Gemcitabine was tolerated in the nude mice tested. The dose of 100mg/kg twice per week did result in a non-sustained drop in weight of the 3 mice. Initial weight loss was observed that became less marked with subsequent dosing. No weight loss exceeded greater than 20% body weight, which would have necessitated subject sacrifice.

5.3.2 AT7519 single agent tolerability

The tolerability of AT7519 treatment was established in non-tumour bearing mice. 3 mice per group were used. The maximum dose and dosing regimen was used as per the experience of Astex pharmaceuticals in other *in vivo* models of AT7519; 7.5mg/kg (Saline vehicle) twice per day for 5 days per 7 day treatment period. The total treatment time was 14 days. Animal sacrifice was performed after the 14 day treatment period, or sooner if animals lost weight of >20% sustained over 72 hours, or if they experienced significant side effects. One animal died on day 6 of treatment. Necropsy performed established an intra-abdominal hemorrhage, therefore this animal died owing to an iatrogenic injury from IP injection rather than a drug effect. There were no other significant side effects or adverse events.

On analysis of the data AT7519 at this dosing was well tolerated with no significant weight loss (shown in figure 5.9).

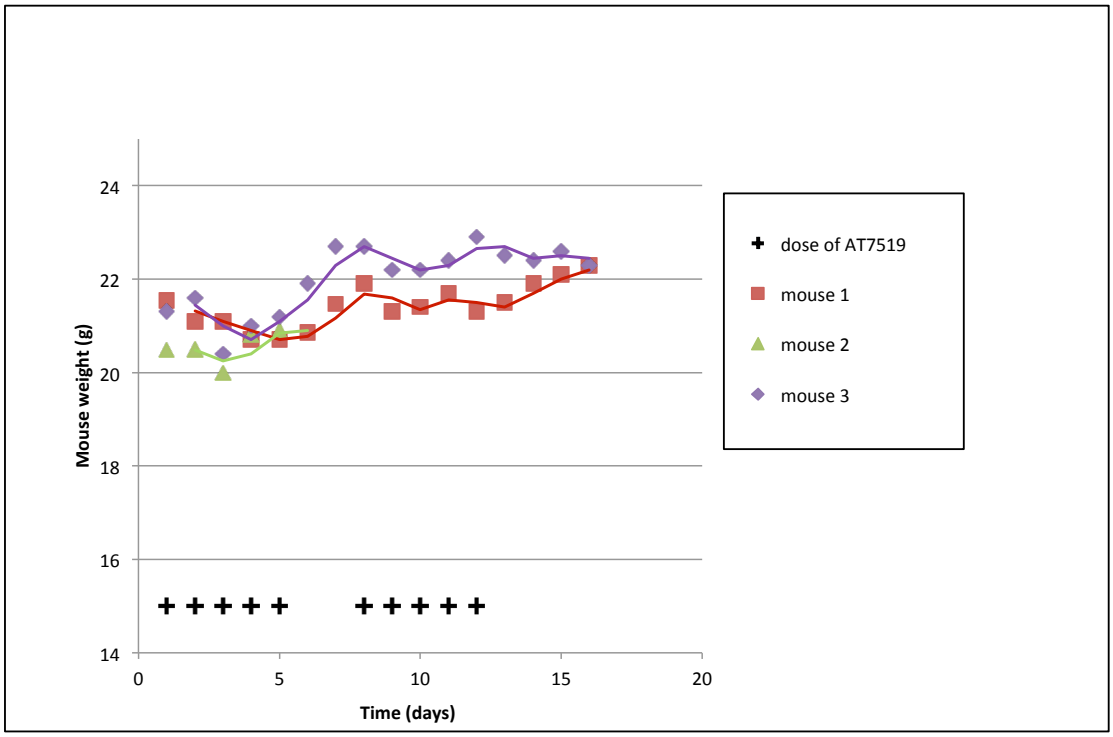


Figure 5.9 A graph showing AT7519 tolerability: The x-axis shows time of experiment in days, the y-axis shows mouse weight in grams. The crosses represent a dose of AT7519. Lines of best fit were produced with a 2-point moving average. 3 mice were dosed at 7.5mg/kg BD for 5 days per 7 day period with AT7519.

CONCLUSION

AT7519 was well tolerated with no weight loss observed at 7.5mg/kg 5 times per week. This dose was deemed appropriate to take forward into single agent efficacy studies.

5.3.3 AT13387 single agent tolerability

The tolerability of AT13387 was established in 3 non-tumour bearing mice. The dose used was 80mg/kg once per week based on the experiments of Astexs pharmaceuticals in other in vivo solid organ malignancy studies. The total treatment time was 14 days. Animal sacrifice was performed after the 14 day treatment period or sooner if animals lost weight of >20% sustained over 72 hours, or experienced significant side effects.

There was no significant weight loss or adverse events experienced with this dosing regime. The dose of 80mg/kg once weekly was tolerated in non tumour bearing mice (data shown in figure 5.10).

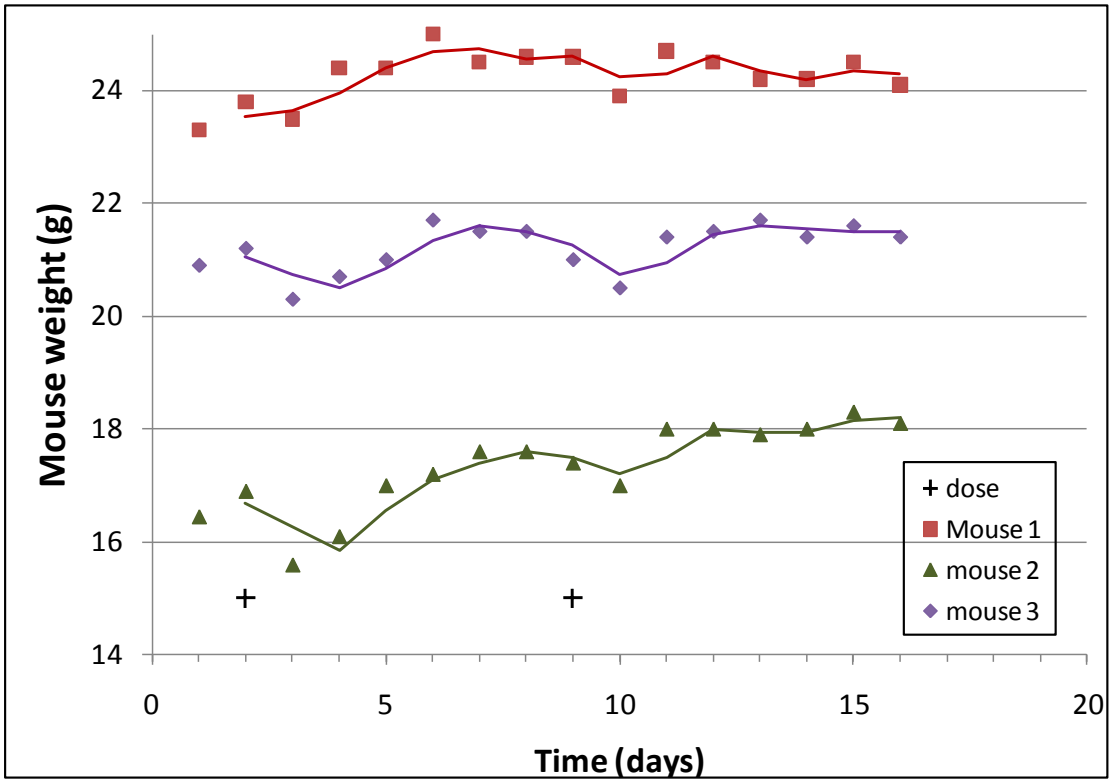


Figure 5.10 A graph showing AT13387 tolerability: the x-axis shows time of experiment in days, the y-axis shows mouse weight in grams. The crosses represent a dose of AT13387. Lines of best fit were produced with a 2 point moving average. 3 mice were dosed at 80mg/kg once weekly with AT7519.

CONCLUSION

AT13387 was well tolerated at 80mg/kg once per week in non-tumour bearing animals therefore was deemed acceptable to take forward to future efficacy studies.

5.4 SINGLE AGENT EFFICACY STUDIES

5.4.1 Gemcitabine single agent efficacy

Gemcitabine was tested as a single agent for efficacy in this xenograft model. Initial efficacy experiments with gemcitabine were performed in tumour bearing mice using the tolerated dose of 100mg/kg twice a week. Treatment was commenced once palpable and measurable tumours were established and tumours were then measured 3 times per week. Each mouse was inoculated with one shoulder tumour. Daily weights and observations were performed. Treatment was continued for up to three weeks. Experiments were performed in Miapaca-2 bearing mice with 6 mice in the treatment group and 6 mice in the saline control group (see table 5.3). Saline controls were given a dosing regime in line with AT7519 treatment.

Groups	No of mice	Dosing regimen
group 1	6	Vehicle control saline 7.5mg/kg twice per day for 5 of 7 days each week
group 2	6	Gemcitabine 100mg/kg twice a week

Table 5.3 A table showing groups, numbers of mice and dosing regime used for the gemcitabine tolerability experiment (the vehicle control dosing regime given was as per AT7519 treatment as this was the maximal dosing used).

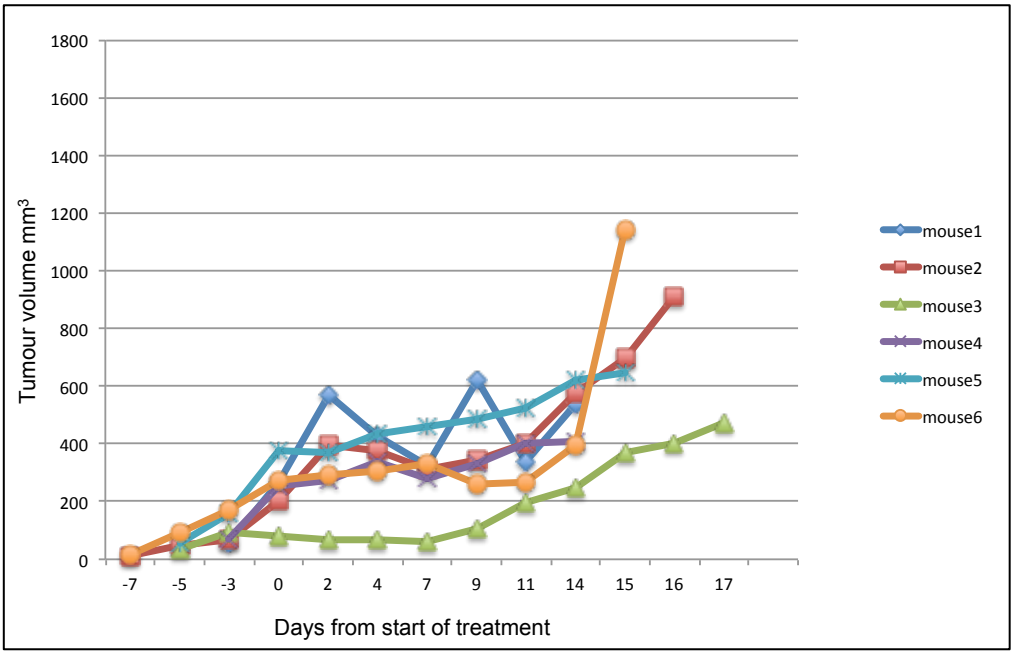


Figure 5.11 A graph showing the tumour volume for 6 mice treated with gemcitabine 100mg/kg twice per week for 3 weeks. The x-axis shows days in relation to starting treatment, the y-axis shows tumour volume in mm^3. Volumes for 6 individual mice are shown.

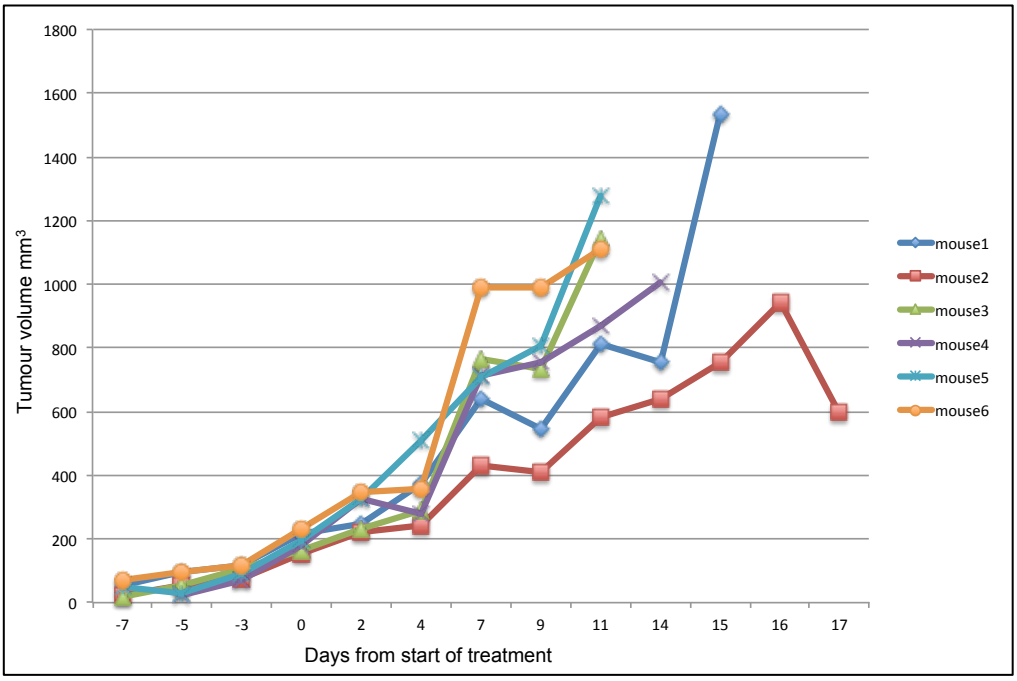


Figure 5.12 A graph showing the tumour volume for 6 mice treated with saline control 7.5mg/kg BD for 5 of 7 days per week for 3 weeks. The x-axis shows days in relation to starting treatment, the y-axis shows tumour volume in mm^3. Volumes for 6 individual mice are shown.

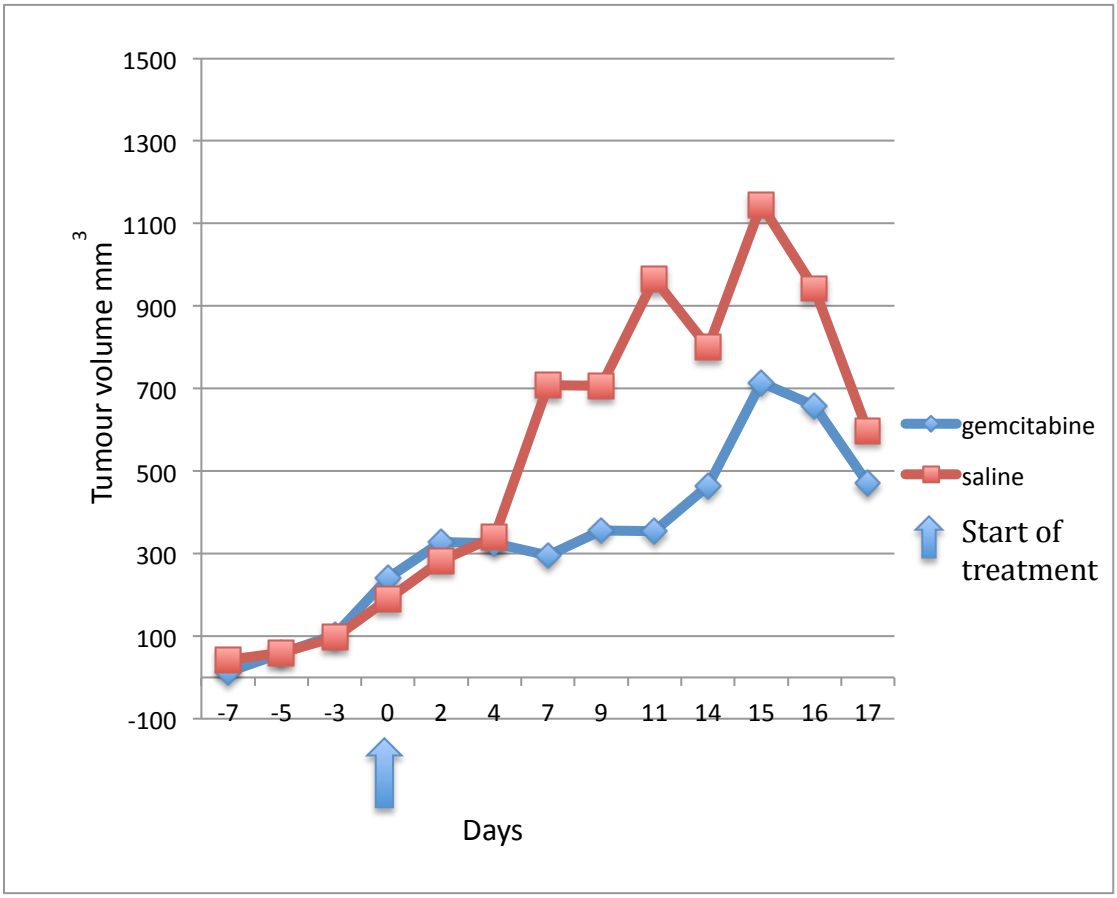


Figure 5.13 A graph showing the total mean tumour volumes for groups 1 and 2 treated with gemcitabine or vehicle control (saline) respectively. The x-axis shows days of treatment, day 0 represents the commencement of treatment. The y-axis shows tumour volume in mm^3.

The tumours grew in an exponential manner and the mean tumours volumes observed in the gemcitabine group were noted to be less than in the matched saline vehicle control (see figure 5.11, 5.12 and 5.13). The data produced was further analysed.

As observed tumour growth occurs in an exponential manner therefore a log regression analysis was performed to examine any difference in the gradients of

growth observed before and after treatment. This further exemplified any effect the agent had as complete tumour regression was not observed.

During the single agent efficacy study, gemcitabine significantly reduced the exponential tumour growth (p=0.0038) compared with the saline vehicle control shown in table 5.4, figure 5.14 and 5.15.

Treatment	Mean coefficient difference	N	P value
Group 1 - Vehicle control saline	0.162	6	0.502
Group 2- Gemcitabine	0.445	6	0.038

Table 5.4 A table of the log regression analysis coefficients mean difference after treatment for each group with p values of significance in difference.

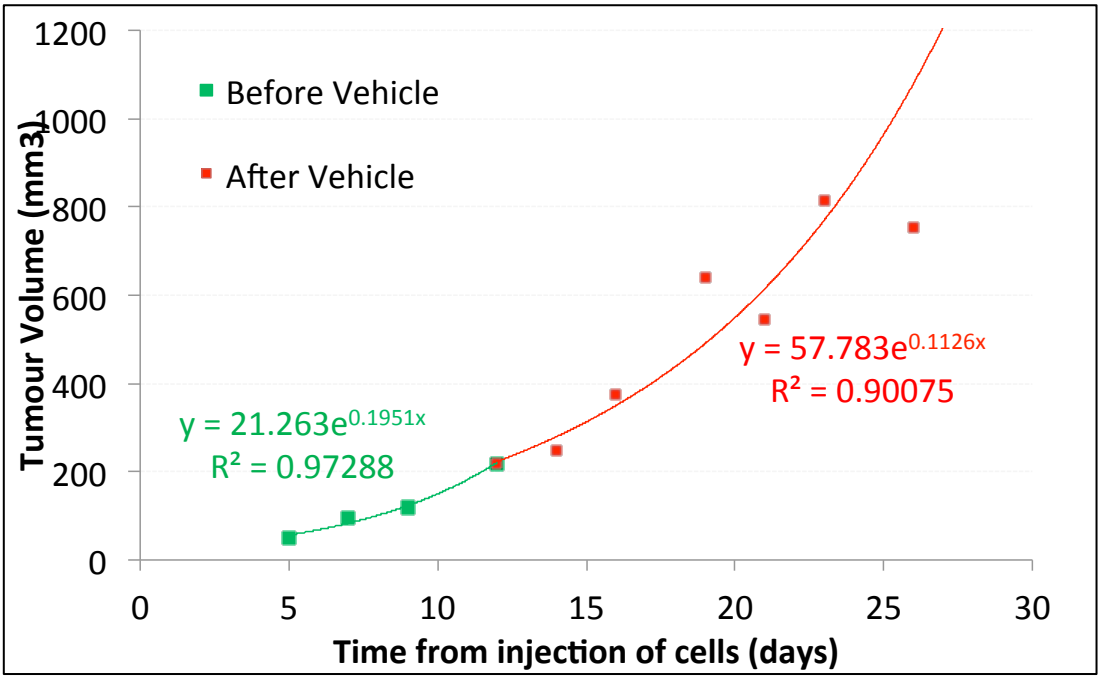


Figure 5.14 A graph showing the tumour volume of a mouse treated with saline vehicle control with log regression analysis co-efficient plotted. The x-axis shows time from injection of tumour cells and the y-axis shows tumour volumes in mm^3.

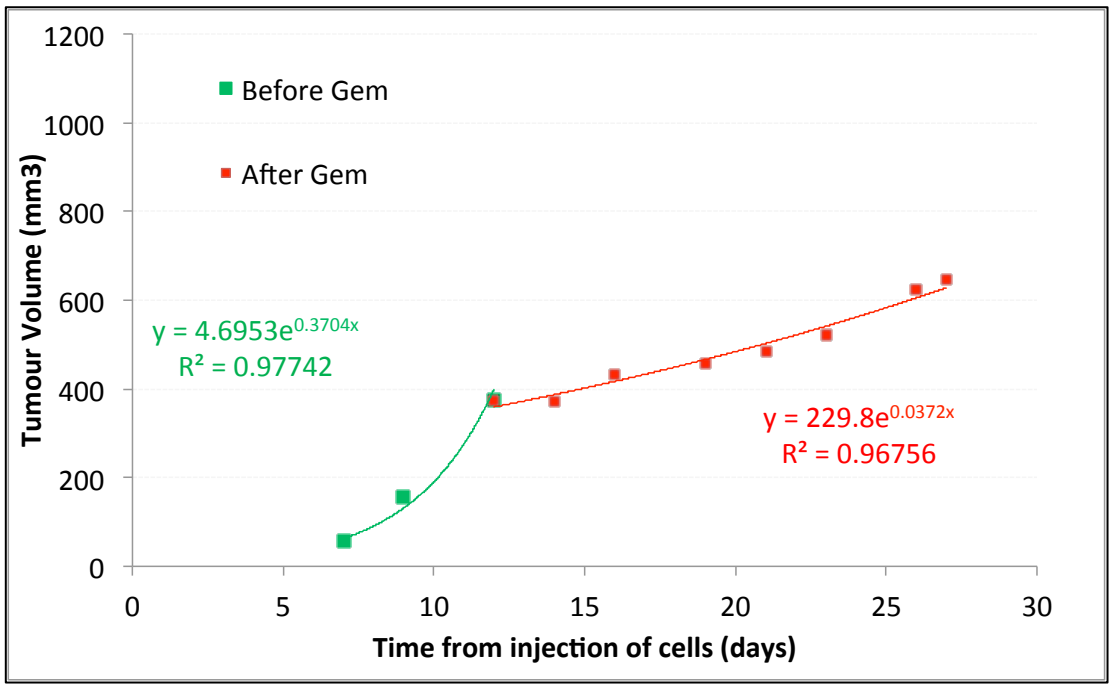


Figure 5.15 A graph showing the tumour volume of a mouse treated with gemcitabine 100mg/kg twice weekly with log regression analysis co-efficient plotted. The x-axis shows time from injection of tumour cells and the y-axis shows tumour volumes in mm^3.

All animals had been sacrificed by day 17 post commencing treatment. In the control group only one animal reached day 17, the 5 other mice were culled earlier owing to tumour volume (this accounts for the fall in tumour volume seen in figure 5.12).

In the gemcitabine treated group one animal reached 17 days, 2 mice were culled secondary to tumour volume on days 14 and 16 and 3 mice were culled owing to sustained weight loss (72hrs) of greater than 20% on days 14 and 15. This accounted for half of the group lost to unacceptable weight loss (again this accounts

for the fall in mean tumour volume rather than treatment exemplified in figures 5.12 and 5.13).

Because of the weight loss observed in non-tumour bearing animals in previous tolerability experiments and the weight loss observed in this first efficacy experiment, two reduced dosing regimens were tested in order to find a moderately efficacious dosing regimen to progress to combination studies.

Experiments for gemcitabine single agent efficacy were performed with gemcitabine with a further dosing experiment with gemcitabine at 50mg/kg twice weekly (table 5.5).

In this single agent efficacy study, gemcitabine significantly reduced the exponential tumour growth ($p=0.0198$) compared with the saline vehicle control shown in table 5.6, figure 5.16 and 5.17. This dose of gemcitabine was deemed to be an efficaous dose to take forward.

Groups	No of mice	Dosing regimen
Group 3	4	Gemcitabine 50mg/kg twice a week (days 1 and 5)
Group 4	4	Vehicle control saline 7.5mg/kg twice per day(days 1-5)

Table 5.5 A table of gemcitabine single agent efficacy studies: groups, numbers of mice and dosing regime used for second experiment with gemcitabine dose reduced to 50mg/kg twice weekly.

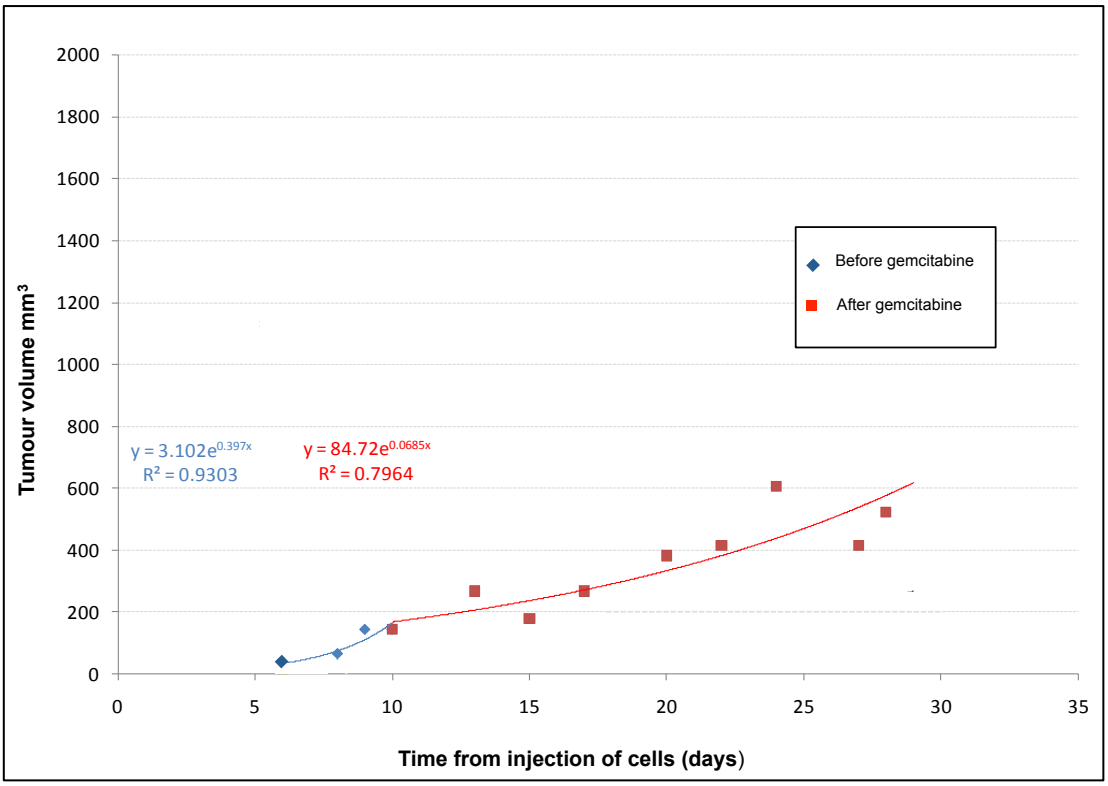


Figure 5.16 A graph showing the tumour volume of a mouse treated with gemcitabine 50mg/kg twice weekly with log regression analysis co-efficient plotted. The Xx-xis shows time from injection of tumour cells and the y-axis shows tumour volumes in mm^3.

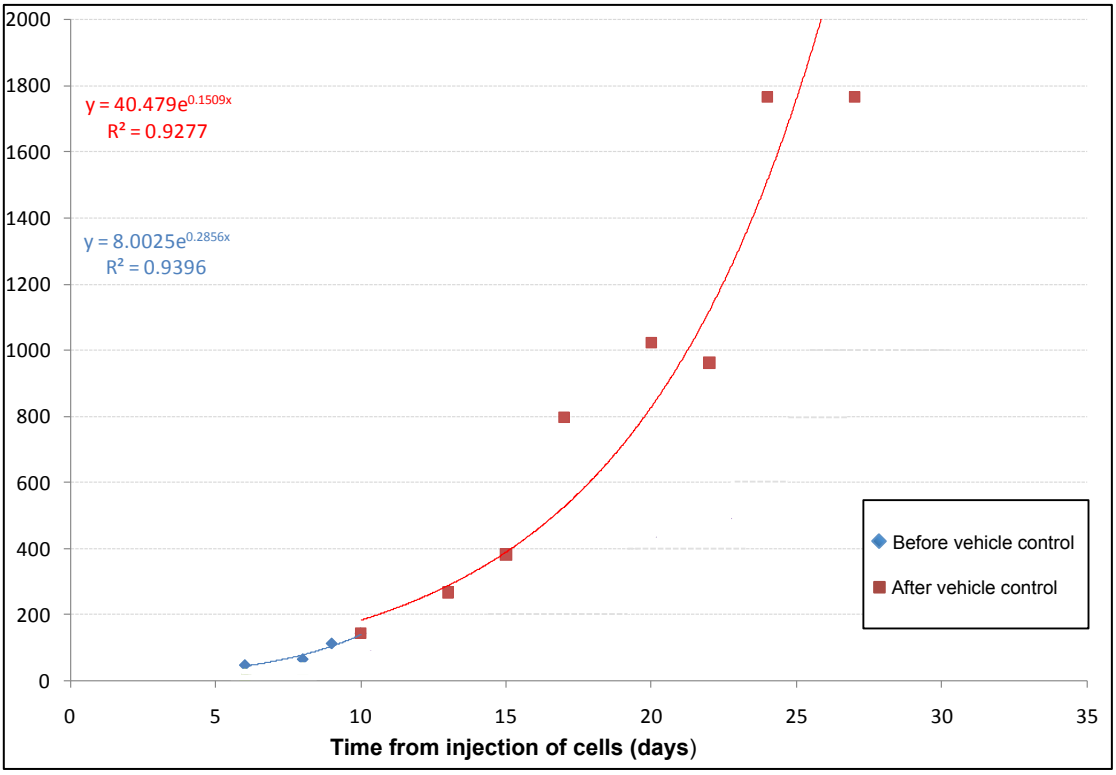


Figure 5.17 A graph showing the tumour volume of a mouse treated with saline vehicle control 7.5mg/kg BD for 5 of 7 days treatment with log regression analysis co-efficient plotted. The x-axis shows time from injection of tumour cells and the y-axis shows tumour volumes in mm^3.

Treatment	Mean coefficient difference	N	P value
Group 3- Gemcitabine 50mg/kg twice weekly	0.162	6	0.0198
Group 4- Vehicle control	0.05	6	-

Table 5.6 A table of the log regression analysis coefficients for groups 3 and 4 (gemcitabine 50mg/kg twice weekly versus vehicle control saline respectively) mean difference after treatment for each group with p values of significance in difference.

In this experiment day 17 post treatment was the experimental endpoint. All 4 controls and 2 mice from the treatment group were culled on day 16 due to tumour volume. The remaining 2 mice in the treatment group were culled on day 17 owing to tumour volume. Importantly no mice were culled secondary to significant weight loss.

CONCLUSION

Gemcitabine was efficacious in this mouse model in reducing exponential tumour growth when compared to saline control (p=0.0198). The initial dosing of gemcitabine at 100mg/kg twice weekly produced unacceptable adverse effects so a lower dose was tested that also proved efficacious. In forward experiments Gemcitabine 50mg/kg was tested in line with the findings.

5.4.2 AT7519 single agent efficacy

The efficacy of AT7519 was examined in this xenograft model. The dosing regime of AT7519 was based on the experience of Astex pharmaceuticals in previous xenograph models they had employed in testing other solid organ tumour types. From tolerability experiments performed it was confirmed the dosing regime would be 7.5mg/kg for 5 days per week (treatment groups shown in table 5.7). There were 6 BALB-c nude mice per group and treatment was commenced 12 days after tumour cell injections, each mouse was inoculated with one shoulder tumour. Daily weights and observations were performed. Once palpable and measurable tumours were established and tumours were then measured 3 times per week. Treatment was continued for up to three weeks.

Groups	No of mice	Dosing regimen
Group 1	6	Vehicle control saline 7.5mg/kg twice daily (days 1-5)
Group 2	6	AT7519 7.5mg/kg twice daily (days 1-5)

Table 5.7 A table showing the AT7519 single agent efficacy study: groups, numbers of mice and dosing regime used.

During the single agent efficacy study, AT7519 significantly reduced the exponential tumour growth (p=0.045) compared with the saline vehicle control shown in table 5.8, figure 5.18 and 5.19.

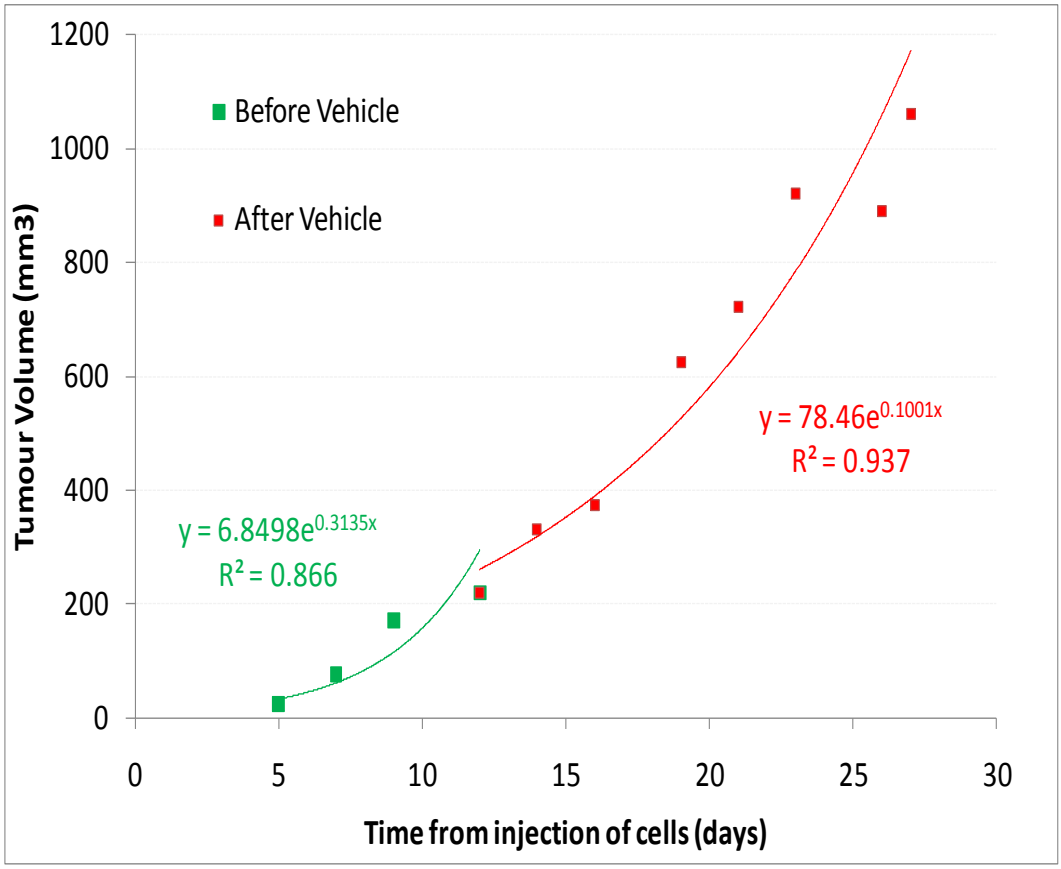


Figure 5.18 A graph showing the tumour volume of a mouse treated with saline vehicle control with the log regression analysis co-efficient plotted. The x-axis shows time from injection of tumour cells and y-axis shows tumour volumes in mm^3.

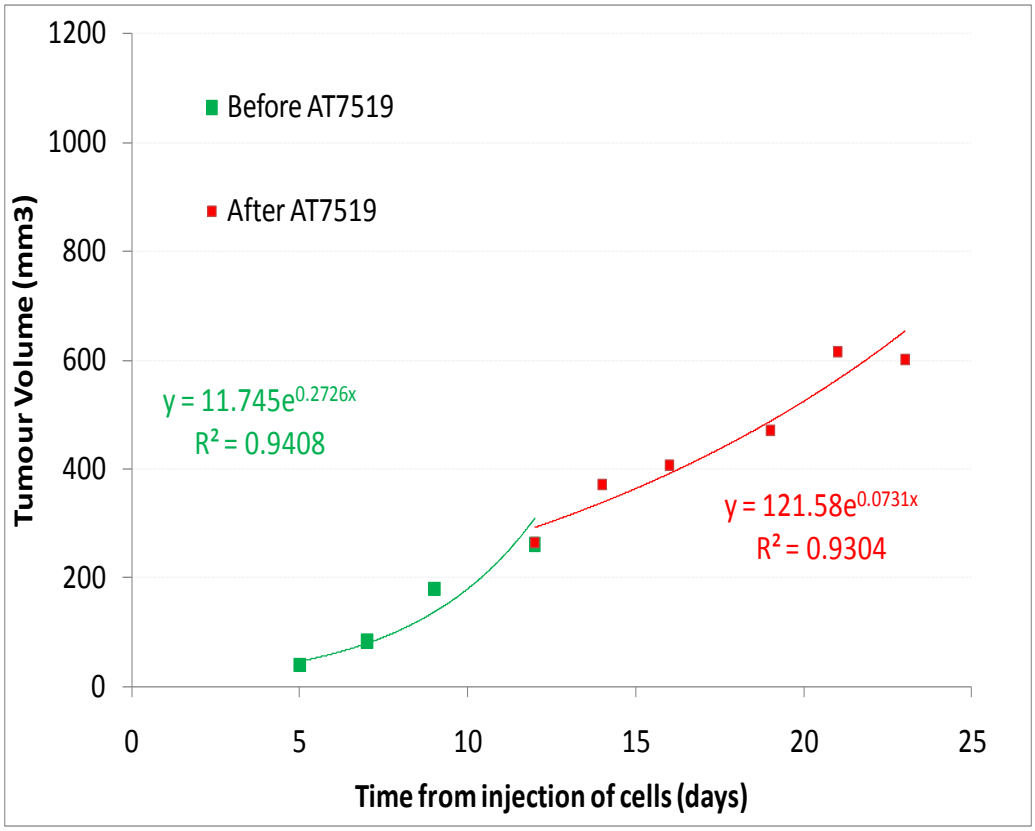


Figure 5.19 A graph showing the tumour volume of a mouse treated with AT7519 7.5mg/kg BD for 5 of 7 day treatment period, with total treatment of up to 3 weeks. The log regression analysis co-efficients are plotted. The x-axis shows time from injection of tumour cells and the y-axis shows tumour volumes in mm^3.

Treatment	Mean coefficient difference	N	P value
Group 1- Vehicle control saline	0.162	6	0.502
Group 2- AT7519	0.29	6	0.045

Table 5.8 A table of the log regression analysis coefficients mean difference after treatment for each group with p values of significance in the difference.

All subjects in the AT7519 group reached at least day 14 of treatment and were culled on the basis of tumour volume, there were no adverse effects or significant weight loss observed (see table 5.9).

Subject	Day of treatment	Reason for cull
Mouse 2	14	Tumour volume
Mouse 5		
Mouse 3	15	Tumour volume
Mouse 4		
Mouse 6		
Mouse 1	18	Experimental end point

Table 5.9 A table of the day and reasoning for cull of mice in the AT7519 treatment group.

CONCLUSION

In this xenograft model of pancreatic cancer AT7519 as a single agent showed a significant reduction in tumour growth compared to vehicle control with no observed significant adverse effects.

5.4.3 AT13387 single agent efficiacy

The efficacy of AT13387 as a single agent was tested in this pancreatic cancer xenograft model. AT13387 was dosed as per the tolerated dosing regimen tested at 80mg/kg once weekly in BALB-c nude tumour bearing mice. Treatment was commenced on day 13 once palpable and measurable tumours were established and tumours were then measured 3 times per week. Each mouse was inoculated with one Miapaca-2 cell shoulder tumour. Daily weights and observations were

performed. Treatment was continued for up to three weeks. There were 6 mice in the treatment group and 6 mice in the cyclodextrin vehicle control group (see table 5.10).

Groups	No of mice	Dosing regimen
Group 1	6	Vehicle control cyclodextrin 80mg/kg once weekly
Group 2	6	AT13387 80mg/kg once weekly

Table 5.10 A table showing the AT13387 single agent efficacy study: groups, numbers of mice and dosing regime used.

There was a trend for the rate of tumour growth as measured by coefficients of an exponential regression plot being reduced by treatment with AT13387 compared to vehicle treated controls (cyclodextrin), this was not statistically significant (p=0.073) (data shown in table 5.11, figure 5.20 and figure 5.21).

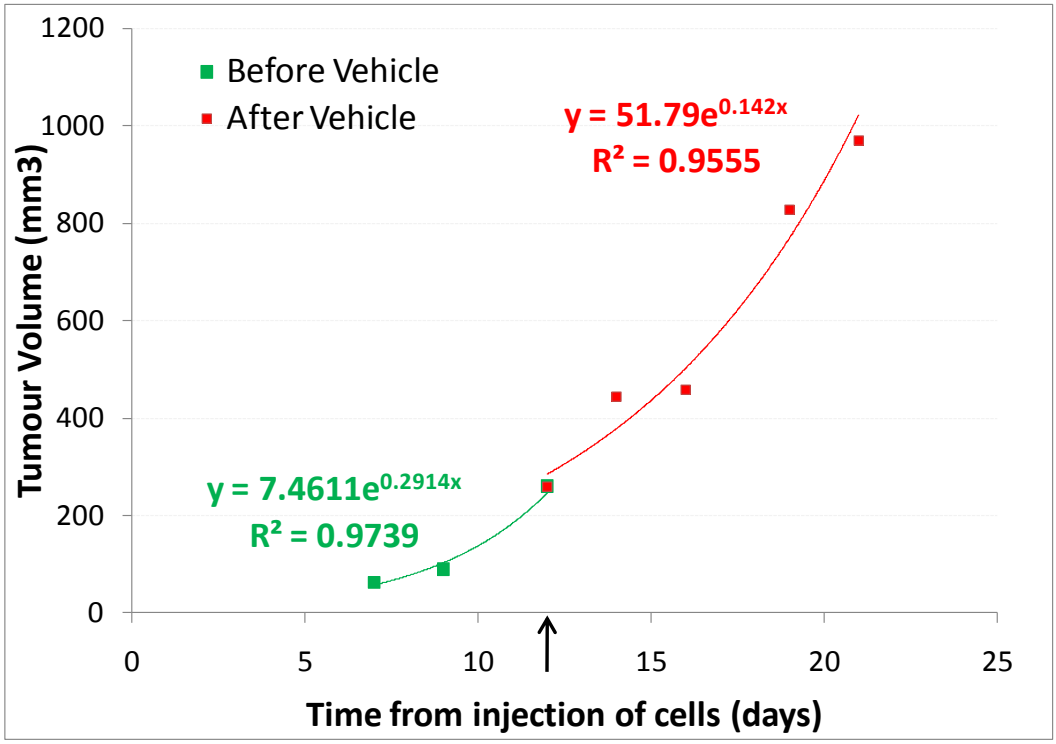


Figure 5.20 A graph showing the tumour volume of mouse treated with cyclodextrin vehicle control with log regression analysis co-efficients plotted. The x-axis shows time from injection of tumour cells and the y-axis shows tumour volumes in mm^3.

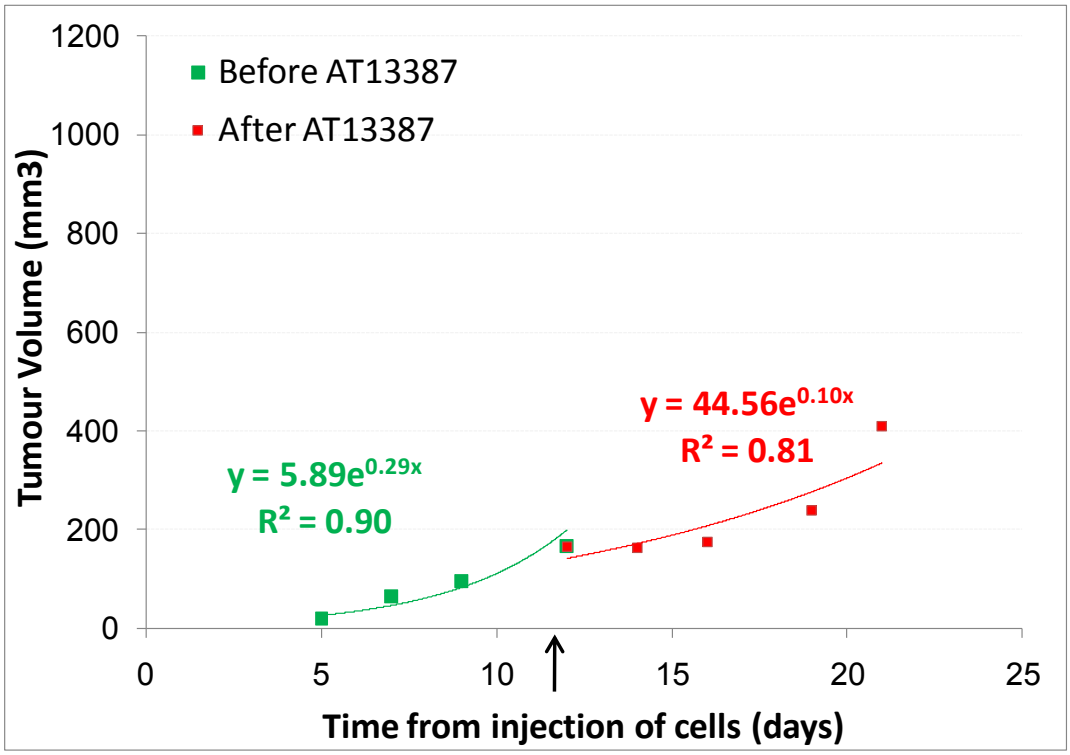


Figure 5.21 A graph showing the tumour volume of a mouse treated with AT13387 80mg/kg once weekly, with total treatment of up to 3 weeks. The log regression analysis co-efficient is plotted. The x-axis shows time from injection of tumour cells and the y-axis shows tumour volumes in mm^3.

Treatment	Mean coefficient difference	N	P value
Group 1- Vehicle control cyclodextrin	0.133	6	0.507
Group 2- AT13387 treatment	0.207	6	0.073

Table 5.11 A table of the log regression analysis coefficients mean difference after treatment for each group with p values of significance in difference for group 1 (cyclodextrin vehicle control) and group 2 (AT13387 treatment).

All subjects were culled by day 16, all controls by day 15. The reasoning for all culls was related to tumour volume. No mice experienced significant weight loss or adverse effects that necessitated cull (see table 5.12).

Subject	Day of treatment	Reason for cull
Mouse 2	14	Tumour volume
Mouse 4		
Mouse 5		
Mouse 6		
Mouse 1	15	Tumour volume
Mouse 3	16	Tumour Volume

Table 5.12 A table of day and reasoning for cull of mice in AT13387 treatment group.

CONCLUSION

AT13387 was tolerated in this pancreatic cancer xenograft model and appeared to slow tumour growth in comparison to vehicle control but this effect was not statistically significant.

5.5 COMBINATION TOLERBILITY STUDIES

After all single agents had been investigated for tolerability and efficacy combination studies were undertaken. Any agent that would potentially be taken forward into human trials would be trial in combination with the current gold standard treatment therefore the investigation of AT7519 and AT13387 in combination with gemcitabine was undertaken for tolerability then efficacy.

5.5.1 AT7519 + gemcitabine combination tolerability

The tolerability of the combination of AT7519 and gemcitabine was tested prior to exploring the efficacy of this combination. The dose of AT7519 remained at 7.5mg/kg twice a day for 5 days per week as this had proved to be tolerable and efficacious in previous studies. The dose of gemcitabine used was 50mg/kg twice weekly as a moderately efficaous dose that was well tolerated in single agent studies (see table 5.13). The tolerability study was performed in non tumour bearing mice for a total period of 2 weeks treatment with 3 mice. Daily weights were performed as well as general health observations. Treatment dosing was

omitted if animals had encountered >15% total body weight loss and terminated if they had sustained (72 hrs.) weight loss >20%.

		Gemcitabine
		50mg/kg twice weekly
AT7519	7.5mg/kg BD (days 1-5)	3 mice

Table 5.13 A table showing the combination tolerability of AT7519 + gemcitabine: Treatments, numbers of mice and dosing regime used

The combination of AT7519 and gemcitabine at the doses tested was tolerated with no significant weight necessitating missed doses or culling of mice (figures 5.22 and 5.23).

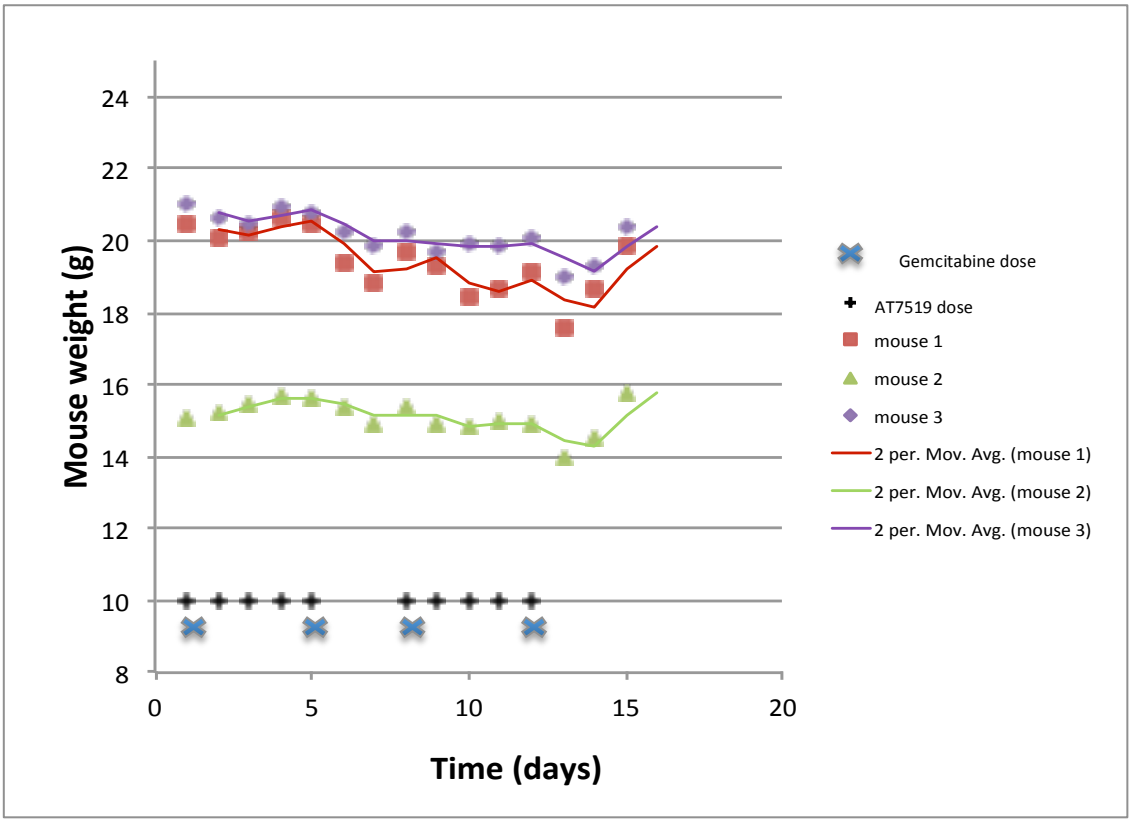


Figure 5.22 A graph showing AT7519 and gemcitabine combination tolerability: the x-axis shows time of experiment in days, the y-axis shows mouse weight in grams. The crosses represent a dose of AT7519, X represents a dose of gemcitabine. Lines of best fit were produced with a 2 point moving average.

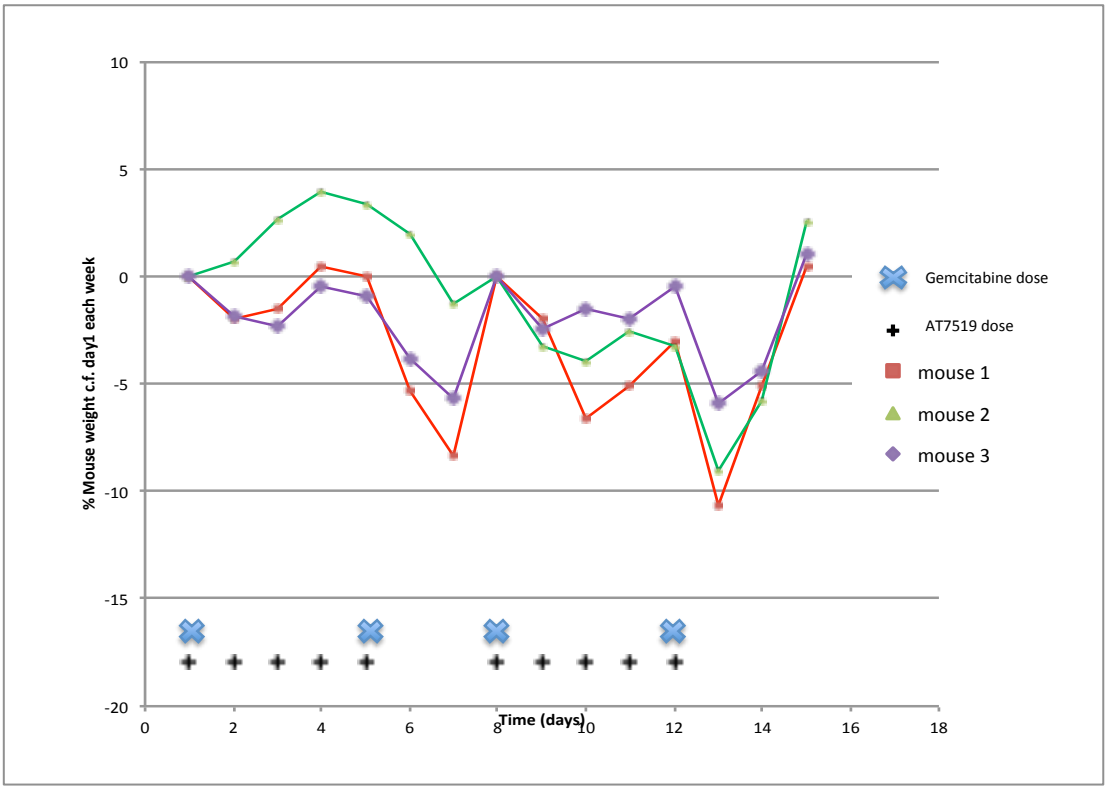


Figure 5.23 A graph showing AT7519 and gemcitabine combination tolerability exemplified by percentage weight loss: the x-axis shows time of experiment in days, the y-axis shows percentage weight loss. The crosses represent a dose of AT7519, the X represents a dose of gemcitabine

CONCLUSION

The combination of AT7519 and gemcitabine at the doses tested was well tolerated in this experiment with no significant weight necessitating missed doses or culling of mice. This dosing regime was taken forward to combination efficacy studies.

5.5.2 AT13387 + gemcitabine combination tolerability [1]

The combination of AT13387 and Gemcitabine was assessed prior to testing combination efficacy of these agents. 3 non tumour bearing mice were dosed for a total of 2 weeks. The dosing of each agent was based on previous tolerability and efficacy studies on these as single agents. AT13387 was dosed at 80mg/kg once weekly (Tuesday) and gemcitabine 50mg/kg twice weekly (Monday and Friday) (table 5.14). Doses were omitted if animals sustained >15% weight loss and the experiment terminated if sustained (72 hrs.) weight loss of >20% was noted.

		Gemcitabine
		50mg/kg twice weekly
AT13387	80mg/kg once weekly	3 mice

Table 5.14 A table showing the combination tolerability of AT13387 + Gemcitabine [1]: Treatments, numbers of mice and dosing regime used.

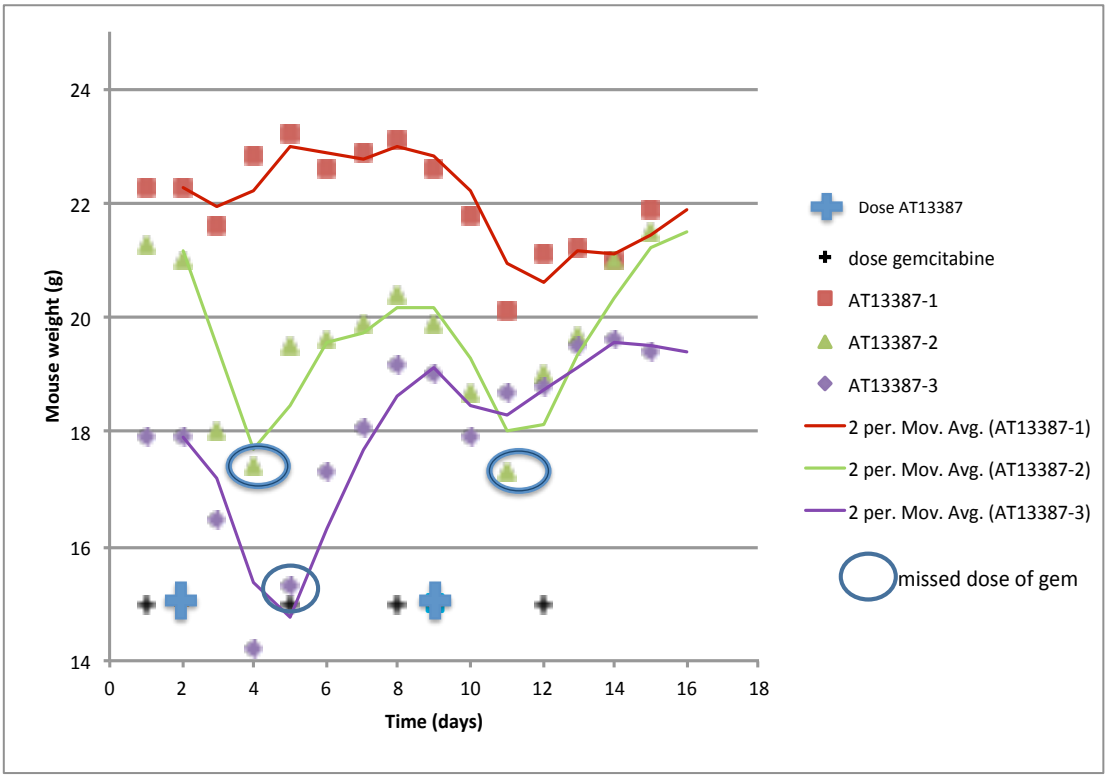


Figure 5.24 A graph showing AT13387 and gemcitabine combination tolerability: the x-axis shows time of experiment in days, the y-axis shows mouse weight in grams. Blue crosses represent a dose of AT1387, black crosses represents a dose of gemcitabine. Lines of best fit were produced with a 2 point moving average.

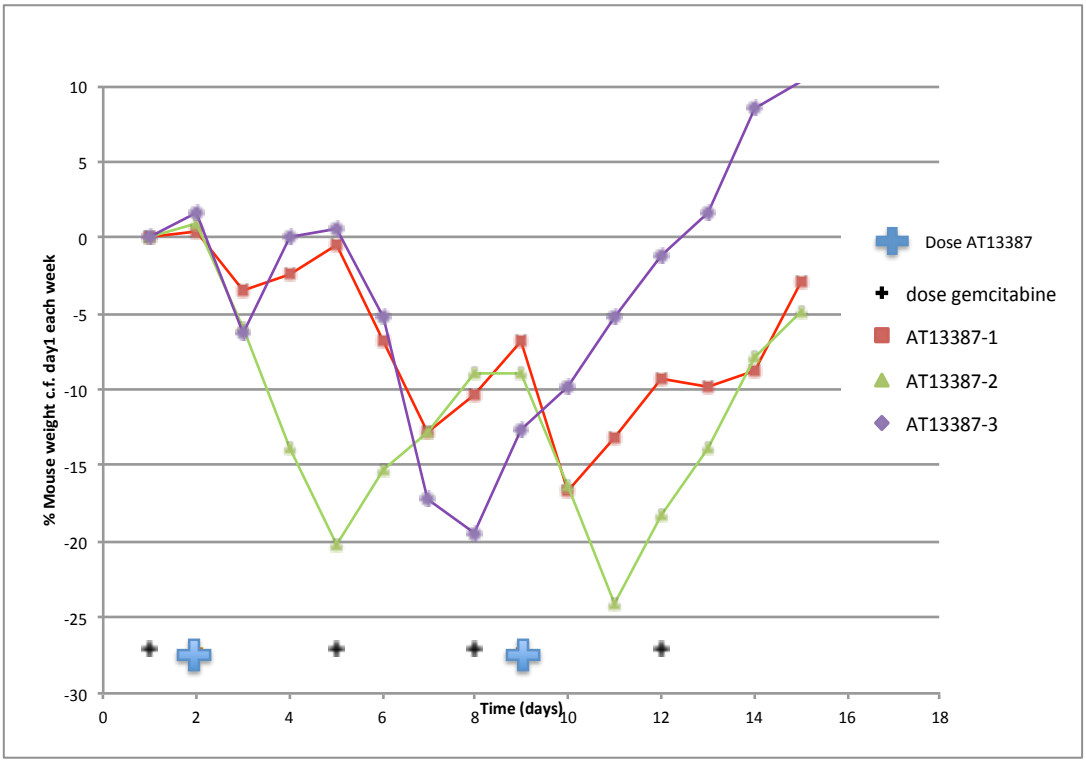


Figure 5.25 A graph showing AT13387 and gemcitabine combination tolerability [1] exemplified by percentage weight loss: the x-axis shows time of experiment in days, the y-axis shows percentage weight loss in each mouse. Blue crosses represent a dose of AT1387, black crosses represents a dose of gemcitabine.

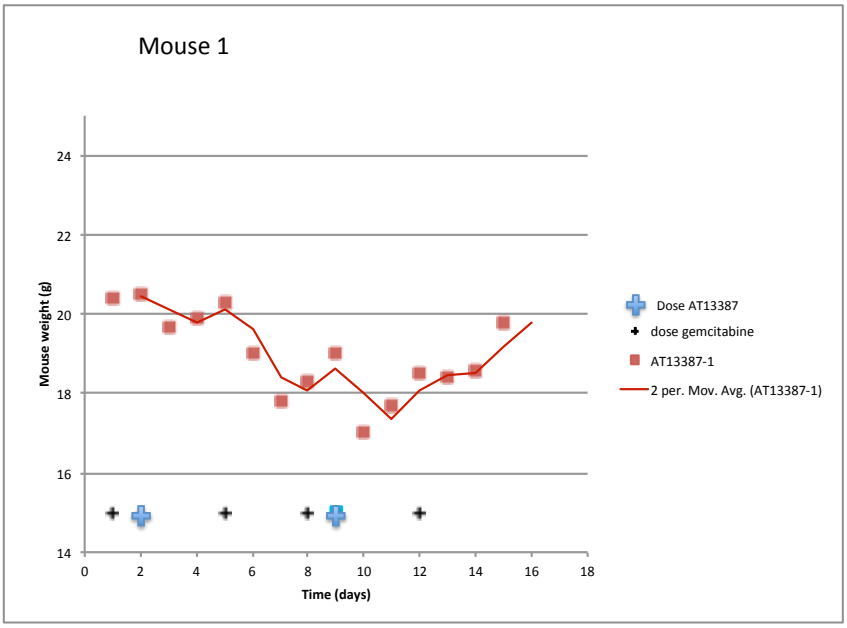


Figure 5.26 A graph showing AT13387 and gemcitabine combination tolerability for mouse 1: the x-axis shows time of experiment in days, the y-axis shows mouse weight in grams. Blue crosses represent a dose of AT1387, black crosses represents a dose of gemcitabine.

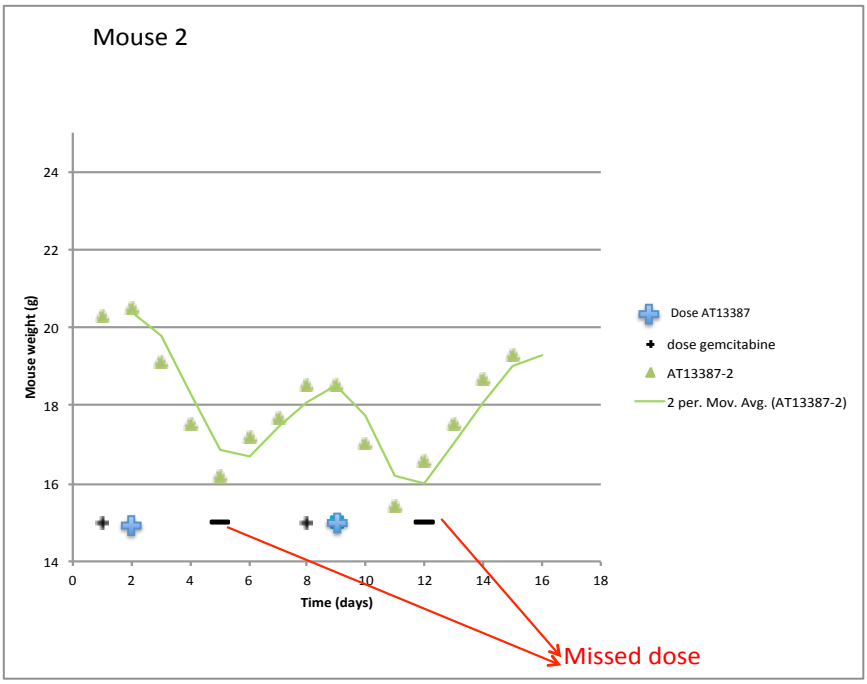


Figure 5.27 A graph showing AT13387 and gemcitabine combination tolerability for mouse 2: the x-axis shows time of experiment in days, the y-axis shows mouse weight in grams. Blue crosses represent a dose of AT1387, black crosses represents a dose of gemcitabine.

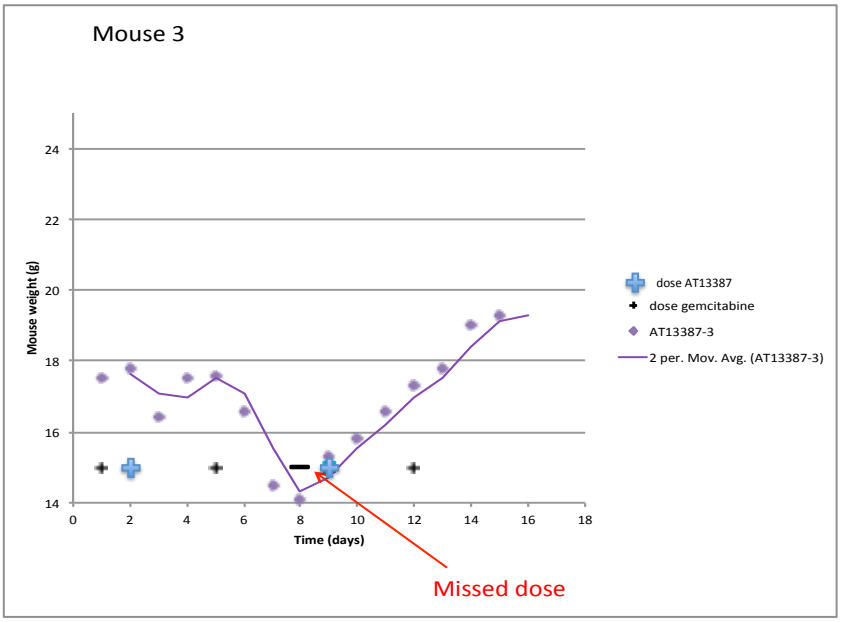


Figure 5.28 A graph showing AT13387 and gemcitabine combination tolerability for mouse 3: the x-axis shows time of experiment in days, the y-axis shows mouse weight in grams. Blue crosses represent a dose of AT1387, black crosses represents a dose of gemcitabine.

During this study significant weight loss was observed that necessitated missing doses. Two gemcitabine doses in 1 animal and one gemcitabine dose in another See figures 5.24 to 5.28.

CONCLUSION

The combination of AT13387 and Gemcitabine at the doses tested was not well tolerated owing to weight loss that necessitated missing doses. In light of this fact this regime would not be taken forward into efficacy studies and a further tolerability study would be undertaken.

5.5.3 AT13387 + gemcitabine combination tolerability [2]

The modifications to the dosing regime for AT13387 and Gemcitabine were considered. Owing to the fact that issues around weight loss were mostly observed with the use of gemcitabine in previous experiments the decision was taken to reduce the dose of gemcitabine to 25mg/kg. 3 non tumour bearing nude mice were used to test this dose modification. The dose of AT13387 remained at 80mg/kg once weekly (see table 5.15).

		Gemcitabine
		25mg/kg twice weekly
AT13387	80mg/kg once weekly	3 mice

Table 5.15 A table showing the combination tolerability AT13387 + Gemcitabine [2]: Treatments, numbers of mice and dosing regime used.

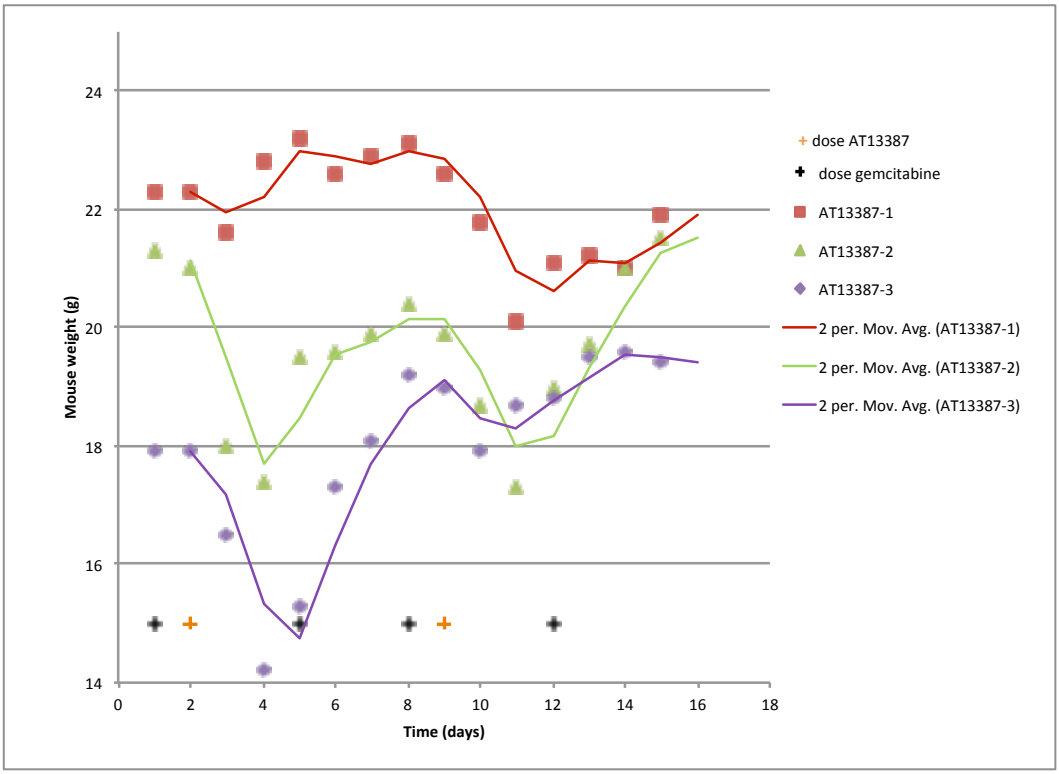


Figure 5.29 A graph showing AT13387 and gemcitabine combination tolerability[2]: the x-axis shows time of experiment in days, the y-axis shows mouse weight in grams. Orange crosses represent a dose of AT1387, black crosses represents a dose of gemcitabine.

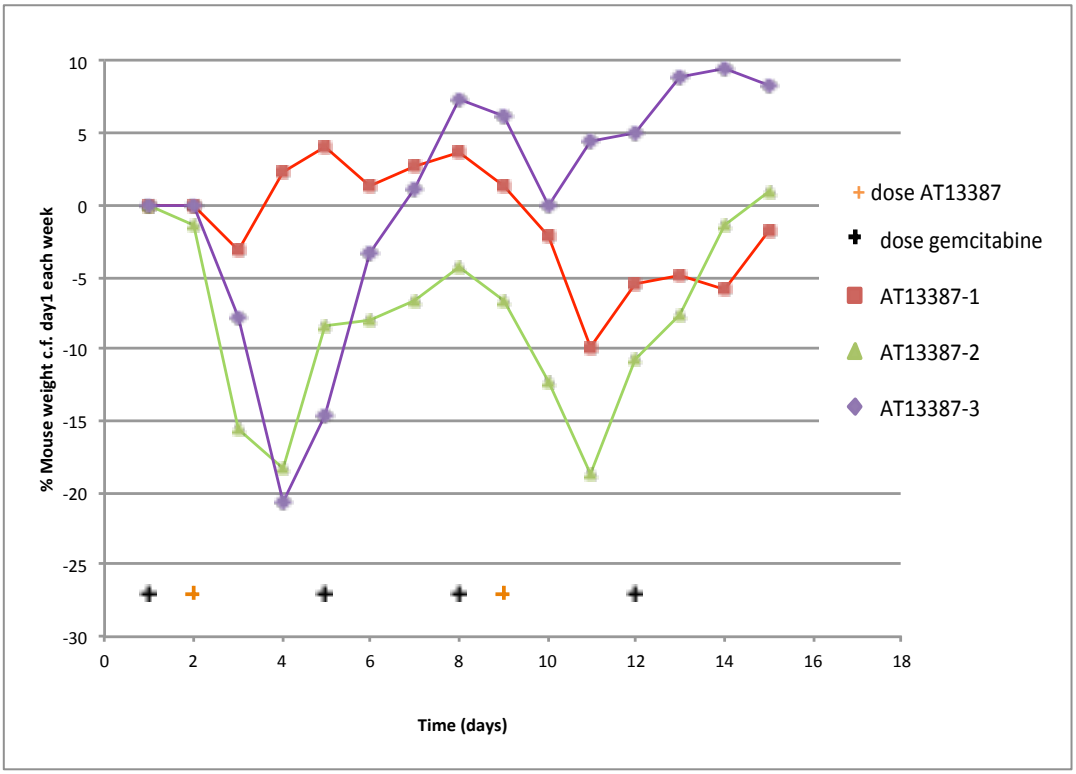


Figure 5.30 A graph showing AT13387 and gemcitabine combination tolerability [2] exemplified by percentage weight loss: the x-axis shows time of experiment in days, the y-axis shows percentage weight loss in each mouse. Orange crosses represent a dose of AT1387, black crosses represents a dose of gemcitabine.

In this study the combination of AT13387 and gemcitabine was better tolerated with only one animal sustaining weight loss that necessitated a missed dose of gemcitabine at the end of the first week (see figure 5.29 and 5.30).

CONCLUSION

The combination of AT13387 at 80mg/kg once weekly and Gemcitabine 25mg/kg twice weekly was better tolerated than previous dosing schedules tested. This dosing regime was deemed acceptable to take forward to efficacy studies of this combination of agents.

5.6 COMBINATION EFFICACY STUDIES

5.6.1 AT7519 + gemcitabine combination efficacy

From combination tolerability studies and single agent efficacy studies this experiment was undertaken with the aim to find the efficacious dosing schedule for the combination of AT7519 and gemcitabine and observe any superior effect to drugs used as single agents. The dosing of AT7519 7.5mg/kg twice daily for 5 day per week and 50mg/kg gemcitabine twice weekly was used. Combined treatment was compared to vehicle control (saline) and single agent treatment with 6 mice per group (see table 5.16 for summary of treatment groups). Dosing was commenced 13 days after tumours were injected subcutaneously and were all measurable in order to enable volume calculations. Treatment was carried out for 3 weeks and the experimental endpoints as previously described adverse events or to the end of the experiment which was terminated 38 post tumour introduction and 25 days post treatment commencing.

Group	Agent	Dose	Number of animals
1	Vehicle 1 (saline)	50mg/kg twice weekly	6
	Vehicle 2 (saline)	7.5mg/kg BD (1-5 days)	
2	Gemcitabine	50mg/kg twice weekly	6
	Vehicle 2 (saline)	7.5mg/kg BD (1-5 days)	
3	Vehicle 1 (saline)	50mg/kg twice weekly	6
	AT7519	7.5mg/kg BD (1-5 days)	
4	Gemcitabine	50mg/kg twice weekly	6
	AT7519	7.5mg/kg BD (1-5 days)	

Table 5.16 A table showing the combination efficacy study of AT7519 + Gemcitabine: Treatments, numbers of mice and dosing regime used.

In the combination efficacy study of AT7519 and gemcitabine a reduction in tumour growth was observed that was superior to vehicle control (p=0.0041) as well as either AT7519 (p=0.0173) or gemcitabine alone (p=0.0611) (data shown in table 5.17 and figures 5.31 and 5.32).

Treatment	Mean coefficient difference	N	P value compared with control	P value compared with gemcitabine	P value compared with AT7519
Saline	0.05	6	-	0.9991	0.9906
Gemcitabine	0.162	6	0.0198	-	0.1477
AT7519	0.114	6	0.029	0.9374	-
AT7519 + Gemcitabine	**0.249**	**6**	**0.0041**	**0.0611**	**0.0173**

Table 5.17 A table of mean coefficient difference for each group before and after treatment with p-values for paired T-test comparing co-efficient change.

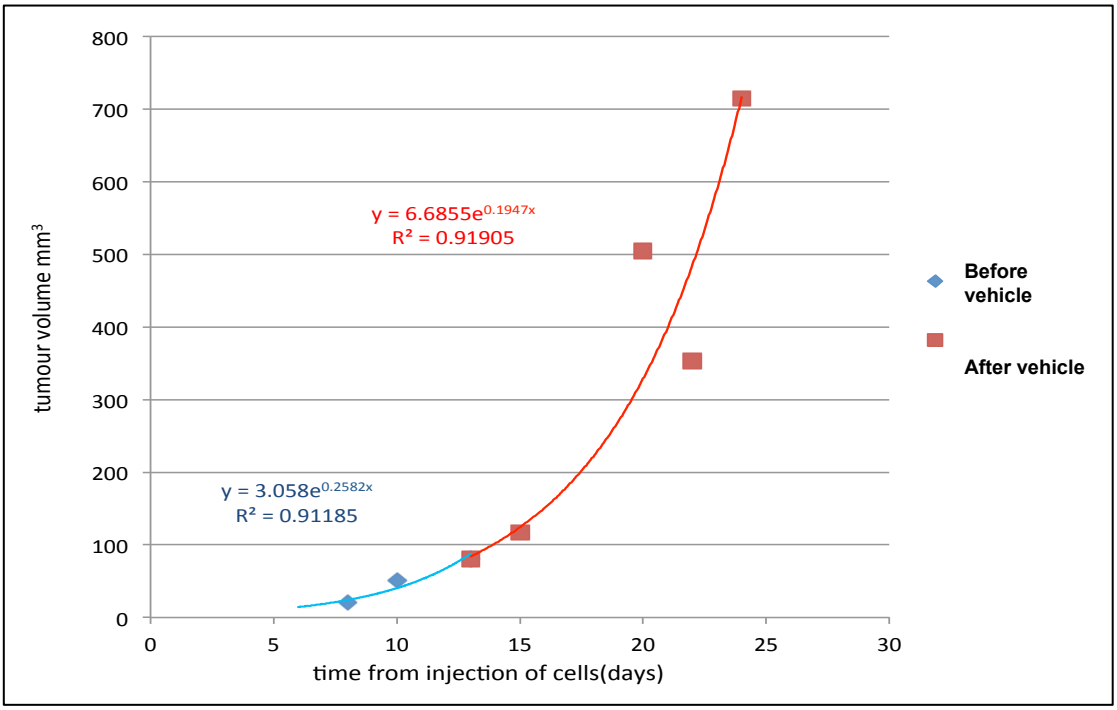


Figure 5.31 A graph showing the tumour volume of a mouse treated with saline vehicle control with log regression analysis co-efficient plotted. The x-axis shows time from injection of tumour cells and the y-axis shows tumour volumes in mm^3.

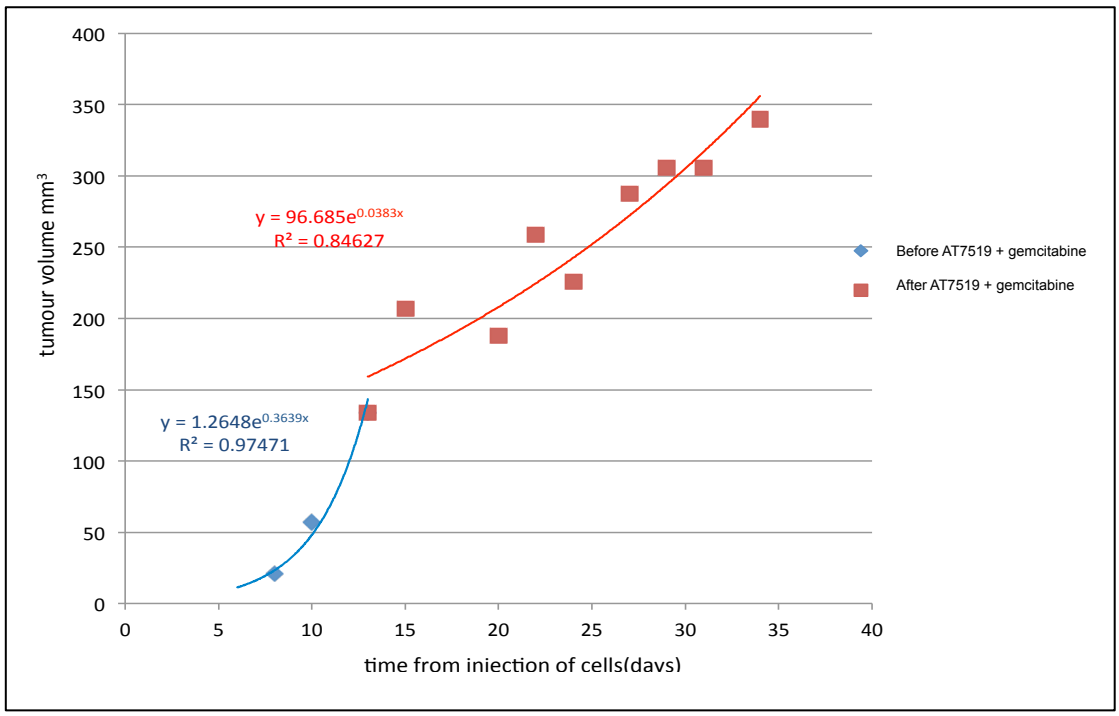


Figure 5.32 A graph showing the tumour volume of a mouse treated with AT7519 (7.5mg/kg BD for 5 of 7 day treatment period) and gemcitabine (50mg/kg twice weekly). Log regression analysis co-efficients are plotted. The x-axis shows time from injection of tumour cells and the y-axis shows tumour volumes in mm^3.

The combination of AT7519 and gemcitabine was relatively well tolerated from day 23 a small number of subjects started to display signs of ill health likely related to the on going tumour burden as non had tumour resolution, this only affected 3 of the combination therapy mice. (Table 5.18 shows why mice were culled prior to the end of the experiment).

Subject	Day	Reason for cull
Mouse 2	23	Tumour volume
Mouse 5	23	General health
Mouse 1	24	General health

Table 5.18 A table of the day and reasoning for cull of mice prior to the end of the experiment. The AT7519 + Gemcitabine treatment group mice only shown.

CONCLUSION

In this xenograft model of pancreatic cancer AT7519 combined with gemcitabine was well tolerated and demonstrated significant reduction in tumour growth compared with vehicle control (p=0.0041), AT7519 as a single agent (p=0.0173) and to a lesser extent gemcitabine as a single agent (p=0.0611).

5.6.2 AT13387 + gemcitabine combination efficacy

Following optimization of the tolerated combination of AT13387 and gemcitabine an efficacy study was undertaken with the aim to demonstrate an efficacious dosing schedule of the combined agents superior to either agent alone.

The dosing of AT13387 780mg/kg once weekly and 25mg/kg gemcitabine twice weekly was used (table 5.19 shows groupings used). In an attempt to avoid significant weight loss dosing of the agents was further spaced out compared with previous experiments. Gemcitabine was administered on Mondays and Saturdays

(previously Monday and Friday) and AT13387 was administered on Tuesdays as previous experiments. Combined treatment was compared to vehicle control (saline or cyclodextrin) and single agent treatment with 6 mice per group. Dosing was commenced 13 days after tumours were injected subcutaneously and were all measurable in order to enable volume calculations. Treatment was carried out for 3 weeks and the experimental endpoints as previously described adverse events or to the end of the experiment which was terminated 33 post tumour introduction and 20 days post treatment commencing.

Group	Agent	Dose	Number of animals
1	Vehicle 1 (saline)	25mg/kg twice weekly	6
	Vehicle 2 (cyclodextrin)	80mg/kg once weekly	
2	Gemcitabine	25mg/kg twice weekly	6
	Vehicle 2 (cyclodextrin)	80mg/kg once weekly	
3	Vehicle 1 (saline)	25mg/kg twice weekly	6
	AT13387	80mg/kg once weekly	
4	Gemcitabine	25mg/kg twice weekly	6
	AT13387	80mg/kg once weekly	

Table 5.19 A table of the AT13387 + gemcitabine combination efficacy study: Treatments, numbers of mice and dosing regime.

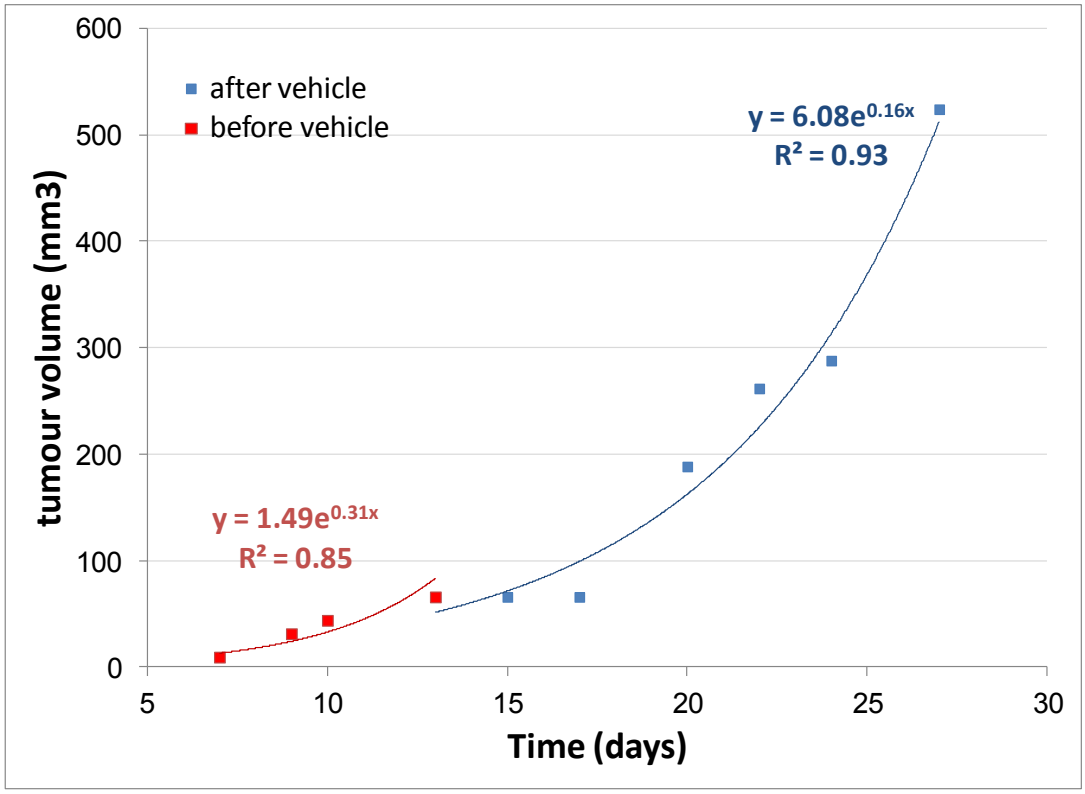


Figure 5.33 A graph showing the tumour volume of a mouse treated with cyclodextrin vehicle control with log regression analysis co-efficient plotted. The x-axis shows time from injection of tumour cells and the y-axis shows tumour volumes in mm^3.

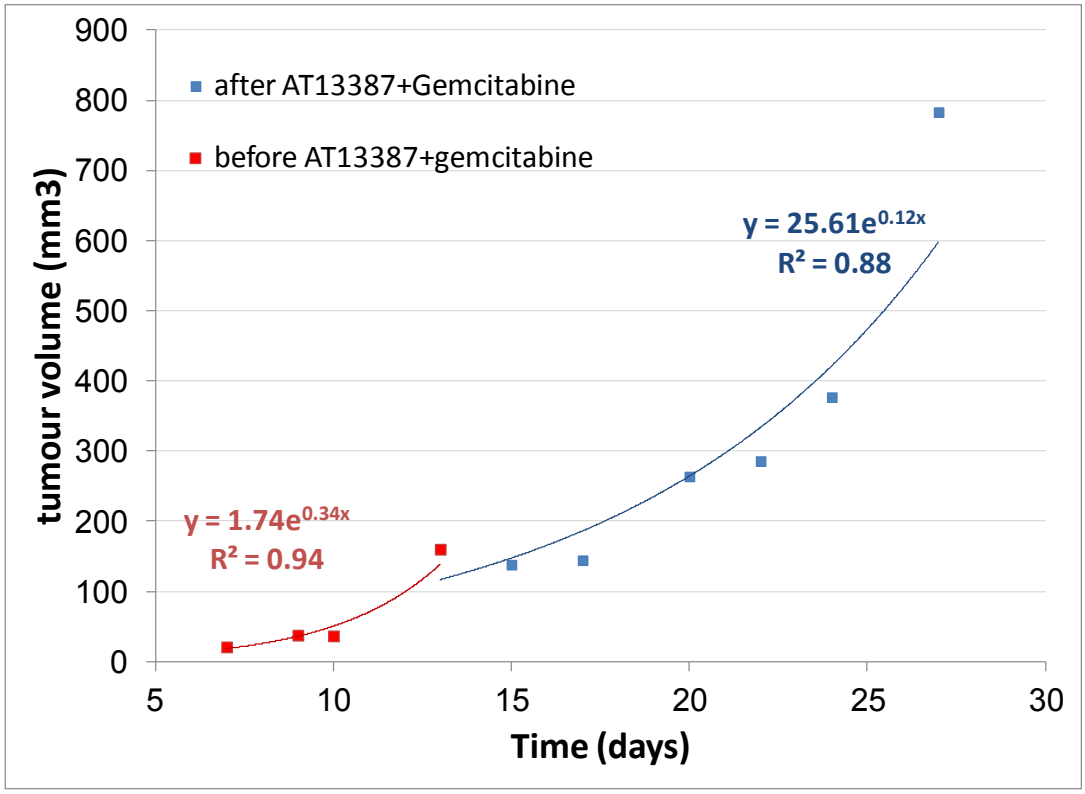


Figure 5.34 A graph showing tumour volume of mouse treated with AT13387 (80mg/kg once weekly) and gemcitabine (25mg/kg twice weekly). Log regression analysis co-efficiens are plotted. The x-axis shows time from injection of tumour cells and the y-axis shows tumour volumes in mm^3.

Following promising single agent results supporting a reduction in tumour growth following AT13387 treatment the compound was tested *in vivo* in combination with gemcitabine. Once weekly dosing of AT13387 at 80mg/kg combined with twice-weekly dosing of gemcitabine at 25mg/kg was tolerated *in vivo*. This schedule was selected as higher doses of gemcitabine combined with AT13387 resulted in substantial weight loss which, although recoverable, resulted in missed dosing at scheduled time points. Tumour growth was significantly slowed with any treatment versus untreated controls (figure 5.33 and 5.34) ($P<0.004$); however, the tolerated

combined dosing did not improve growth inhibition over either AT13387 or gemcitabine administered alone (figure 5.35).

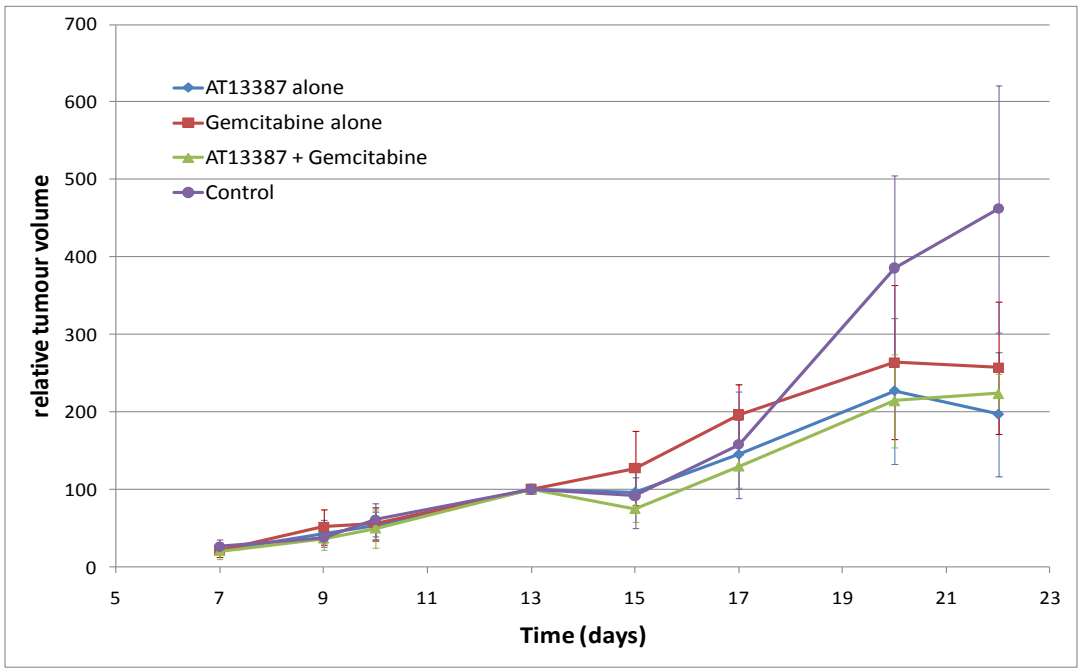


Figure 5.35 A graph showing the relative tumour volumes in mice treated with Saline vehicle control, Gemcitabine alone, AT13387 alone and Gemcitabine + AT13387. Treatment commenced on day 13. The x-axis shows time in days from injection of cells and the y-axis shows the relative tumour volumes.

Adverse events were encountered during this experiment. Three animals died unexpectedly and one was sacrificed owing to ill health (2 had combination therapy and 2 single agent AT13387). Necropsy was performed on the animals that died unexpectedly showed ascites and dilated bowel in all 3 animals.

During the experiments 3 doses of gemcitabine and one dose of AT13387 was missed in the combination experiment and one dosed was missed in the gemcitabine single agent group all owing to weight loss >15%.

CONCLUSION

AT13387 and gemcitabine reduced tumour growth significantly compared to controls but was no better than single agents in slowing this growth. Furthermore there were issues around tolerability and adverse events that did not support a synergistic or even additive effect of these agents.

CHAPTER 6: DISCUSSION

In this thesis I have shown the combination of gemcitabine and novel agents targeting CDK and HSP can be made to improve response in models of PDAC.

While the targeting specific molecules and pathways has been previously shown to offer benefits for pancreatic cancer patients, this is a complex disease with multiple genetic and protein defects. Targeting pathways such as that of CDK inhibition and HSP90 inhibition offers opportunities to target multiple pathways including some of those established as important in pancreatic cancer.

6.1 AT7519 – A NOVEL CDK INHIBITOR

6.1.1 Inhibiton of cell growth *in vitro*

During this research project the in vitro effects of AT7519 against pancreatic cancer cells lines was established. AT7519 demonstrated ability to induce growth inhibition across a range of pancreatic cancer cell lines. The effect of AT7519 was explored in standard lines and an in-house developed gemcitabine resistant line. In light of the known chemoresistance demonstrated to gemcitabine the effects of novel agents in this cell line were important. It was observed that AT7519 demonstrated ability to inhibit growth in this resistant line furthermore looking at

the relative IC50 values the resistant line was more sensitive to AT7519 then the parent line, the converse situation was true when these cell lines were treated with gemcitabine.

6.1.2 The issue of gemcitabine resistance in PDAC

Gemcitabine resistance is an important issue to overcome in PDAC and the mechanisms are being uncovered as *in vitro* and *in vivo* study is taken into this area. The action of gemcitabine is dependent on its uptake into cells via transporters including hENT1 and 2 (human equilibrative nucleoside transporter 1 and 2) and hCNT1 and 3 (human concentrative nucleoside transporters 1 and 3) then intracellular metabolism via dCK (enzyme deoxycytidine kinase) leads to an active metabolite that is taken into the nucleus to cause DNA damage and ultimately apoptosis (Mini, Nobili et al. 2006; Wong, Soo et al. 2009).

The resistance to gemcitabine seen in PDAC can be attributed both to its ability to reach the target cells and the cellular events leading to gemcitabine metabolism including the levels of transporters and enzymes aforementioned (Mackey, Mani et al. 1998; Andersson, Aho et al. 2009). It has also been suggested that loss of function of p53 may play a role in this resistance.

Clearly from this *in vitro* investigation AT7519, as a CDK inhibitor, was not subject to the same cellular blocks to its activity and if anything was more effective in the gemcitabine resistant line, the mechanisms for this remain unknown but this offers promise for AT7519 as a potential second line agent in patients who have not responded to gemcitabine therapy or AT7519 to be given in combination with gemcitabine to attempt to overcome the resistance currently seen. In previous *in vitro* studies of AT7519, colorectal cell lines with mutant p53 were noted to be sensitive to AT7519 treatment, perhaps this goes some way to explaining why the gemcitabine resistant line investigated here was more sensitive to AT7519(Squires, Feltell et al. 2009).

6.1.3 Cell cycle analysis of AT7519

As a single agent AT7519 resulted in a clear cell cycle arrest in the G2/M phase, with a marked reduction of cells in G0/1 and S phases. Its activity observed here in pancreatic cancer cell lines is consistent with previous investigations of this agent in other solid organ tumour types including colorectal models (Squires, Feltell et al. 2009). It is also in line with inhibition of substrates of CDKs 1 and 2, supporting AT7519 as a potent inhibitor of these CDKs(Nyberg, Michelson et al. 2002; Donzelli and Draetta 2003). When AT7519 and gemcitabine were combined the effects observed were more in line with that of gemcitabine as a single agent, specifically S phase accumulation. Gemcitabine is an S phase active drug and evidence suggests it exerts a G1/S phase arrest (Huang and Plunkett 1995). It has been noted however, in some cell lines that this does not lead to an immediate apoptosis (Cappella,

Tomasoni et al. 2001). Although these cells do experience blocks at G2/M and G1 again and have been observed to subsequently be highly sensitive to programmed cell death. This has important implications in combination therapy as this suggests that additional cell cycle agents maybe beneficial on these already sensitized cells. The S phase accumulation observed was more marked using this combination compared to the actions of gemcitabine alone as a single agent. This raises two possibilities; AT7519 may be enhancing the cell cycle effects of gemcitabine or exerting effects on the S phase checkpoint.

From literature evidence the S phase checkpoint is in part dependent on CDK 2 activity, therefore AT7519 may be exerting its effects on CDK2 activity more notably in these cells that have been arrested by gemcitabine activity (Bartek and Lukas 2003; Bartek, Lukas et al. 2004). The cell cycle analysis reported here shows that AT7519 has different effects to that of gemcitabine. In combination the two drugs increased apoptosis. As these agents have effects at different points in the cell cycle it is clear that dosing schedule has a potentially important role.

6.1.4 Cytotoxic effects and apoptosis induced by AT7519

AT7519 ability to induce apoptosis was demonstrated by caspase 3 activity. It was noted *in vitro* the increased caspase-3 activity was seen between 16 and 48 hours after treatment with AT7519. In this data presented it is worth noting that an exposure time of more than 24 hours i.e. equivalent or greater than one cell cycle,

was required to observe significant apoptosis. Perhaps it is at certain points in the cell cycle that AT7519 needs to be present in order to induce apoptosis. Previous investigation of AT7519 in Myeloma cell lines also noted apoptosis between 12 and 48 hours and this was also observed in colorectal cell lines(Squires, Feltell et al. 2009; Santo, Vallet et al. 2010).

6.1.5 Combination treatment of AT7519 with standard therapy

The importance of scheduling AT7519 with gemcitabine to allow both agents to take maximal effect was further demonstrated by isobolar analysis. When AT7519 and gemcitabine were combined in an isobolar model the initial experiments produced results consistent with antagonism. It appeared that AT7519 prevented gemcitabine from having its expected effect. This can be explained by the fact that Gemcitabine as a nucleoside analogue needs cells to be actively dividing in order to have its effect, when cells are treated with AT7519 the G2/M block prevents this. Interestingly this same antagonism was not indicated in the cell cycle analysis where gemcitabine still seemed to be having an effect, in fact the S phase accumulation noted was more marked when the drugs were combined. In light of the antagonism observed sequential addition of the agents was tested. The results showed that when cells were treated with gemcitabine initially then the combination of gemcitabine and AT7519 at least an additive effect was achieved. Although this is a crude demonstration it does highlight the importance of agent scheduling to allow both agents to have their maximal effect. This could be taken further with trailing different drug schedules but the value of this in an *in vitro* model is questionable in

light of the fact in human dosing further factors play a role in drug delivery such as tumour microenvironment therefore potentially make this effect less important.

6.1.6 Current literature on combination treatment

The combination of gemcitabine and other novel CDK inhibitors has been reported in the literature in the pre clinical setting. Flavopiridol is a selective CDK inhibitor with a profile similar to that of AT7519 (inhibition of CDK 1,2,4 and 7) and has demonstrated activity in a number of tumour types *in vitro* and in xenograft models (Bible and Kaufmann 1996; Carlson, Dubay et al. 1996). Its combination therapy with gemcitabine and the importance of drug sequencing was investigated in the pre-clinical setting. It was noted that gemcitabine followed by flavopiridol resulted in the maximum antitumour effect and the pro apoptotic effects of gemcitabine were enhanced. The reverse combination did not show any superiority over either agent alone (Jung, Motwani et al. 2001). On the basis of this clinical trails have commenced with this combination using the drug sequencing indicated by pre clinical studies (Fekrazad, Verschraegen et al. 2010).

6.1.7 Downstream effects of AT7519

When downstream markers were investigated the effects of AT7519 on key pathways in the cell cycle was demonstrated and again the importance of dose scheduling investigated. The decreased levels of cdc2 after treatment with AT7519 supports the G2/M block that was observed in the cell cycle work. When AT7519

was given in combination with Gemcitabine this decrease was less marked and when gemcitabine was given prior to AT7519 no effect on cdc2 levels were noted. This can be explained by the effect of gemcitabine causing an S phase arrest, supported by the cell cycle activity of AT7519 in combination with gemcitabine were this marked accumulation of cells in S phase occurred. Rb phosphorylation, which is catalaysed by CDKs (namely CDK2) was decreased, this action being in line with the expected CDK inhibition AT7519 induced. The fact that AT7519 is active in inhibiting the phosphorylation of Rb, a critical point that governs the G1/S transition and therefore subsequent DNA replication, offers promise in its potent action and a potential synergy with gemcitabine that is having effects at this point in the cell cycle. This was also observed in phospho pp1-a and phospho NPM proving that AT7519 has an effect on CDK inhibition as all of these proteins have phosphorylation that is catalysed by CDKs. The decreased levels of Phospho NPM were also investigated when cells were treated with the combination of AT7519 and gemcitabine and this effect was again present by more marked with combination treatment. This perhaps is explained by the fact that NPM is a nuclear residing protein, in the nucleolus or cytoplasm of the nucleus and perhaps the effects of gemcitabine and its transport within the nucleus inhibit its phosphorylation further.

In targeting multiple pathways general CDK inhibitors such as AT7519 provide a promising therapeutic option excerpting both direct cell cycle and transcription effects(Malumbres 2012), evidence of actions on some of these known aberrant pathways have already been investigated. Fry et al demonstrated the activity of PD

000332991 on tumour xenografts, this CDK inhibitor specifically targets CDK 4 and 6 and therefore the downstream phosphorylation of Rb(Fry, Harvey et al. 2004).

The observations on downstream markers showed AT7519 is having an effect on the cell cycle although these were not significant enough at the expected IC50 doses of AT7519 to establish any protein that could be used as biomarkers for treatment at this stage, changes in NPM were observed in a phase I study of AT7519 but not in a consistent fashion that would highlight it as a potential biomarker for activity (Mahadevan, Plummer et al. 2011).

6.1.8 *In vivo* investigation of AT7519

In a murine model AT7519 was investigated as a single agent and in combination with gemcitabine. As a single agent in the xenograft model AT7519 was well tolerated and there was no observed adverse events or significant weight loss in no tumour bearing mice. In the subsequent efficacy study of pancreatic cancer cell line tumour bearing mice AT7519 remained well tolerated and proved to be efficacious. There was a significant reduction in tumour growth compared to control. The effects of AT7519 on tumour xenografts in colorectal cell lines showed more marked evidence of apoptosis where regression of tumours was noted with AT7519 treatment(Squires, Feltell et al. 2009), the effects observed in this model were more in line with the current evidence for CDK inhibitors in Xenograft models where reduction in growth with no noted regressions have been noted (Patel, Senderowicz

et al. 1998; McClue, Blake et al. 2002). More recently a novel CDK inhibitor Dinaciclib was demonstrated to inhibit pancreatic cancer progression in a murine model and its CDK 5 inhibition hypothesized to affect Ras-mediated Ral activation(Feldmann, Mishra et al. 2011).

AT7519 was also tested in a xenograft model in combination with gemcitabine. This study would to be pivotal in supporting the use of AT7519 in pancreatic cancer as any novel agent would be tested alongside the standard therapy if it were advanced to human trials, therefore proving compatibility or synergy with the standard therapy would be mandatory. In this murine model the combination of AT7519 and Gemcitabine was well tolerated and proved to be efficacious. The combination treated mice had a significant reduction in tumour growth compared to control and AT7519 as a single agent, there was also a trend for superiority over gemcitabine alone though this was not quite statistically significant ($p = 0.0611$). The results gained here are similar to that of other work done on CDK inhibition in pancreatic cancer models. P276 (a CDK 4 inhibitor) was tested in combination with gemcitabine in a comparable animal model. In this model combination treatment with P276 and gemcitabine was superior to control and either as a single agent and no significant toxicity was encountered (Subramaniam, Periyasamy et al. 2012). In this model PANC-1 (kras mutated line relatively resistant to gemcitabine) was used and neither the novel agent or gemcitabine caused any significant tumour growth suppression as a single agent. This raises an interesting discussion point regarding the effects seen in our model. If it were repeated in a more gemcitabine resistant

line such as PANC-1 or SUIT-2 GR the effects of the combination therapy may be more marked.

6.1.9 The relevance of CDK inhibtion to PDAC

Gemcitabine resistance in pancreatic cancer remains an important issue that limits current therapeutic results. The inability of gemcitabine to induce apoptosis in a large number of pancreatic tumours is multifactorial but the intimate relationship between the cell cycle and programmed cell death cannot be dismissed for example the central role of p53 in both these processes which upon its loss of normal function leads to cell cycle control loss and alterations in the pathway to apoptosis conferring gemcitabine resistance(Galmarini, Clarke et al. 2002).

Regarding the use of CDK inhibitors in pancreatic cancer the important question remains that which CDKs or range of CDKs should be target and would provide the optimum therapeutic option for this disease. Clearly it seems attractive to targets multiple CDKs as tumours cells by their nature are unable to stop at predetermined points in the cell cycle because of the loss of integrity of these checkpoints. Targeting multiple CDKs would recapture these stop check points that limit the tumour cells ability to pass through the cell cycle and may then allow induction of apoptosis. This would obviously be more assured if multiple targets were used rather then a single target and potentially single point within the cell cycle. This far most of the combined studies of chemotherapeutics and CDK inhibitors has used

pan-CDK inhibitors such as flavopiridol although focus have shifted toward developing compounds that are more potent and selective in their action (Dickson and Schwartz 2009). The combination of chemotherpaturitcs and CDK inhibitors that have an effect on CDK1 and 2 (like AT7519) may be the most desirable combinations owing to the synergistic effects noted in the literature with DNA damage causing chemotherapeutic agents (Ira, Pellicioli et al. 2004; Maude and Enders 2005). As the knowledge on the genetic basis of pancreatic adenocarcinoma becomes clearer this question will be more readily answered, certainly the effects demonstrated by AT7519 offer promise and suggest that at least partly this selective CDK inhibitor offers a useful therapeutic option as an adjunct to standard therapy. The information to date on CDK inhibitors support the notion that using these agents in combination rather than monotherapy offers the most desirable therapeutic avenue and the data generated from this work supports this.

Collectively these results support the notion that AT7519 could be a useful agent in combination with gemcitabine to improve therapy in pancreatic cancer and overcome some of the cell cycle proteins involved in the resistance of pancreatic cancers to gemcitabine. The anti-proliferative effects seen in vitro were further supported by in vivo efficacy of this combined therapy that was well tolerated.

6.1.10 Suggested further work

AT7519 has demonstrated that it has effects against PDAC in the pre clinical setting. As an agent that has been investigated in early phase clinical trials in other solid organ tumours it would be appropriate to take this agent forward in PDAC, in this pre clinical model its efficacy has been limited but this may be enhanced by further biomarker work to attempt to identify subgroups of patients that may benefit most from this agent, patients with innate or acquired gemcitabine resistance may derive benefit from the addition of AT7519 in the advanced cancer setting.

6.2 AT13387 – A NOVEL HSP90 INHIBITOR

6.2.1 Inhibition of cell growth *in vitro*

In this study the effects of AT13387 were examined *in vitro* and *in vivo* both as a single agent and in combination with gemcitabine. AT13387 induced growth inhibition across a range of pancreatic cancer cell lines at the nanomolar level. The IC50 values obtained in this study are comparable to the values that have been obtained in other cancer types (Graham, Curry et al. 2012). The sensitivity of a gemcitabine resistant cell line was comparable to that of the parent line but more importantly the absolute IC50 value obtained for this gemcitabine resistant cells line was significantly less than that of gemcitabine treatment. This offered encouraging results that AT13387 may be a potential novel therapy to overcome the problem of gemcitabine resistance in pancreatic cancer. The sensitivity of the parent line and

gemcitabine resistant line were similar suggesting that AT13387 was not subject to the same underlying reasons for gemcitabine resistance that have been displayed and chemoresistance may not be an issue for this agent. One advantage of HSP90 inhibitors may be that HSP90 inhibition leads to degradation of multiple oncogenic client proteins and effects multiple pathways in tumourgenesis so that resistance to their action may not be an issue.

When cells were treated with AT13387 then treatment was removed cell regrowth occurred. This suggested AT13387 was having a cytostatic rather than cytotoxic effect. This effect of AT13387 is notably different to other HSP90 inhibitors but may not necessarily be a disadvantage when used in the setting of combination therapy. The ability of AT13387 to leave the cell in a quiescent state may then allow the opportunity for chemotherapeutics to have an effect and give the final push to induce apoptosis.

6.2.2 Cell cycle effects of AT13387

The cell cycle effects of AT13387 were demonstrated with an increase of cells in G0/G1 and G2/M phases after treatment. In combination with gemcitabine the S phase accumulation of cells exhibited by gemcitabine treatment alone was documented. The multiple pathways and proteins targeted by HSP90 inhibition may account for there being no specific point in the cell cycle where cells accumulate. The encouraging data from this cell cycle analysis is that despite the

multiple points in the cell cycle where AT13387 suspends cells it does not inhibit gemcitabine having an effect as the same effects of gemcitabine as a single agent are observed in combination with AT13387.

6.2.3 Investigation of apoptosis in AT13387

When apoptosis was investigated it was demonstrated beyond 40 hours after treatment with AT13387. AT13387 does not appear to produce apoptosis at an early stage, this is converse to the observations with other HSP90 inhibitors (Kim, Kang et al. 2003) and the actions of AT13387 observed in other tumour lines (astex pharmaceuticals, data not published). The fact that AT13387 interferes with protein folding and subsequent cell division on a number of levels may suggest that the ability to push the cell forward to apoptosis takes some time, perhaps pancreatic cancer cells are more resistant to this final push to programmed cell death.

6.2.4 Combination treatment with standard therapy

While inhibition of HSP90 may prove therapeutic as a single agent it is also likely to find use in combination with other agents as the effect of a number of chemotoxic agents has been reported to be enhanced by other HSP90 inhibitors such as 17AAG or geldanacycim (Burkitt, Magee et al. 2007). It has also been suggested that the global effect of HSP90 inhibition to potentiate the action of other agents may act

through mechanisms quite distinct from those employed when acting as a single agent.

The effects of combining AT13387 and gemcitabine in vitro in an isobolar analysis did not yield any clear results in these experiments, though these combination studies did allow some observations. There was a trend for enhanced AT13387 in the presence of gemcitabine and perhaps this is more striking as in these experiments AT13387 did not reach its IC50 as a single agent but did in the presence of gemcitabine. Gemcitabine acts on the cell in S phase causing irreversible DNA damage but as suggested previously this may not occur on the first cell cycle that gemcitabine comes into contact with the cell, perhaps the initial insult of gemcitabine treatment makes the cells more vulnerable to the actions of AT13387 in subsequent cell cycles and therefore further investigation of this combination with sequential drug addition may shed further light on the situation.

6.2.5 Downstream effects of AT13387

AT13387 induced inhibition of HSP90 was evident from examination of client proteins. Both Akt and phospho Akt levels were reduced after treatment with AT13387, this effect was less marked when cells had been treated with gemcitabine prior to AT13387 treatment. This is in line with the expected action of HSP90 inhibition as suppression of survival pathways such as p13/Akt is often observed as one of the earliest effects of HSP90 inhibition (Graham, Curry et al. 2012). The fact

that after gemcitabine treatment the effects were less prominent suggests cells were already sustaining the DNA damaged caused by gemcitabine treatment and a lesser effect of AT13387 was noted. The same effect was true on phospho S6 when AT13387 was given as a single agent as decreased levels were observed. Combining AT13387 with gemcitabine appeared to enhance this effect irrespective of the order in which the agents were given. From literature evidence gemcitabine does not effect S6 phosphorylation therefore this enhanced effect on phospho S6 offers encouraging results and suggests the addition of AT13387 opens this pathway to inhibit cellular proliferation more so in combination treatment (Martin-Liberal, Gil-Martin et al. 2014)

Client proteins involved in the important MAPK cascade were also examined after treatment with AT13387. Raf-1 levels were decreased in cells treated with AT13387. This effect was enhanced with the combination treatment with gemcitabine. In addition to these above many cell cycle regulatory proteins are affected by HSP90 inhibitors, including CDK4, and in this study CDK4 levels were reduced following treatment with AT13387, this effect was more marked and noted at lower doses of AT13387 when gemcitabine treatment was used in combination, irrespective of dosing regime. These data support the notion that AT13387 induced HSP90 client protein degradation that was not inhibited or antagonized by combination therapy with gemcitabine.

Treatment with AT13387 resulted in significant up regulation of HSP70 both as a single agent and combined with gemcitabine, as a co-chaperone to HSP90 inhibition this is in line with actions of HSP90 inhibition previously recognized in the literature. This effect was not inhibited by combination treatment or pre treatment with gemcitabine. The overexpression of HSP70 relates to the heat shock response induced y this inhibitor and it is worth noting this could effect the agents anti-cancer activity as overexpression of HSP70 could induce pro-survival and anti-apoptotic effects. This may go some way to explaining the only modest tumour effects observed in the xenograft model.

6.2.6 *In vivo* investigation of AT13387

The effects of AT13387 as a single agent in a mouse xenograft model initially suggested potentially promising effects. The drug was well tolerated as a single agent and did result in slowed tumour growth compared to control though this was not statistically significant. When the agent was combined with gemcitabine the results were unfortunately less favorable. The combination of AT13387 and gemcitabine was no better at slowing tumour growth than either agent alone and significant issues were encountered with tolerability and toxicity of these agents combined.

The tumour half-life of AT13387 has been demonstrated prolonged in a lung cancer cell line xenograft model. After a single dose tumour half-life was 65 hours

compared to around 4 hours in plasma/blood (Graham, Curry et al. 2012). The effects on downstream markers were prolonged compared to other HSP90 inhibitors such as 17-AAG. In this same model tumour growth was significantly reduced compared to controls. An advantage of this prolonged tumour half-life is not only the prolonged tumour retention and potential window of action for AT13387 but also the need for less frequent dosing therefore reducing the systemic effects/toxicities that treatment may produce.

6.2.7 Current literature in the *in vivo* setting

Dose limiting toxicity in HSP90 xenograft models and human studies has been previously noted, mostly relating to gastrointestinal and liver toxicity (Banerji, Walton et al. 2005; Sequist, Gettinger et al. 2010). It is not surprising bearing in mind the mechanism of action of HSP90 inhibitors that global effects are seen frequently. In this model AT13387 as a s ingle agent was well tolerated perhaps owing to the prolonged tumour half-life therefore less frequent dosing regime that was employed. Although dose limiting toxicity was experienced in this model in combined treatment of AT13387 and gemcitabine in phase I studies of AT13387 alone these have not been encountered at the same dosing levels offering encouragement that in human trials the same issues may not be encountered in combination therapy. Even at higher doses of AT13387 in humans the toxicities encountered were grade 1-2(Do, Speranza et al. 2015). The fact remains that HSP90 inhibition signaling pathways have not been fully explored hence could be more complicated than expected and systemic toxicities difficult to overcome.

A number of HSP90 inhibitors have been reported to inhibit the growth of tumours in pancreatic xenograft models alone (Moser, Lang et al. 2012) or in combination with other agents (Cao, Jia et al. 2008; Li, Zhang et al. 2011). This supports the potential use of HSP90 inhibition in pancreatic cancer treatment. Here AT13387 as a single agent was well tolerated and showed a modest effect on tumour growth. It was disappointing that the combined treatment of AT13387 and gemcitabine in this xenograft model showed no improvement over either single agent alone. Unfortunately poor tolerability of the combined regime resulted in a reduce gemcitabine dosage to that initial planned for combination studies which may have impacted on the efficacy in this model. Each agent alone produced some modest weight loss but the combined therapy resulted in an additive effect in relation to this side effect that impacted on dosing and survival in the model. Further investigations may reveal more favorable drug scheduling to improve tolerability and efficacy of this combination therapy.

6.2.8 AT13387 with gemcitabine

This study sought to investigate AT13387 in combination with the current gold standard treatment for pancreatic cancer, gemcitabine. Combined treatment with gemcitabine and AT13387 apparently resulted in no greater effect than either single agent in both the in vitro and in vivo setting. It appears that there may be some

compensatory or competing mechanisms that precludes any additive or synergistic effect of combining these agents. It was notable during in vitro studies that the up-stream protein kinase Raf-1 was not noticeably elevated by gemcitabine treatment and if anything pre-treatment with gemcitabine increased the effect of AT13387 to reduce raf-1 protein levels further then the decrease seen with AT13387 alone. Further investigation is required to examine the effects on other members of the pathway including MEK. These data suggest a potential avenue of investigation of combining MEK inhibitors in this regime. There are a number of MEK inhibitors under investigation including a number of phase I/II clinical trials examining MEK inhibitors in combination with gemcitabine (Wong 2009; Trujillo 2011).

Although this data would not support adding AT13387 to standard gemcitabine therapy, the continued activity of AT13887 in the gemcitabine resistant Suit-2GR cells indicates that this agent may be of benefit in patients who have not responded to gemcitabine treatment and possibly in combination with other alternative agents that may enhance its activity such as MEK inhibitors. The fact remains that pancreatic cancer contains multiple molecular aberrations and targeting HSP90 and its multiple downstream effects may offer some benefit in improving outcomes in this complex disease.

6.2.9 Suggested further work

With the above in mind further work at this stage would not revolve around taking this agent forward in the clinical setting but further pre clinical work n AT13387 in combination with standard therapy. Further investigation of this agent in combination with cell lines that are resistant to gemcitabine therapy may offer a sub group that would have an enhanced benefit from this agent, biomarker discovery would be required in order to progress to clinical trials to properly identify this group of patients. The combination of gemcitabine and AT13387 in the in vivo setting seemed to induce toxicity that limited observations on efficacy of this combination, it may be beneficial to investigate this agent along with newer agents becoming standard therapy in PDAC such as capecitabine to investigate any enhanced effects of these agents in combination.

CHAPTER 7: CONCLUSION

In human malignancies irregularities in the cell cycle are commonly encountered. The knowledge of these abnormalities has lead to the rationale of developing this class of agents, CDK inhibitors. Single agent activity of these agents may be limited but their combination with other cyctotoxics offers a promising avenue of therapy not only to overcome issues with tumour resistance to current treatments but also to develop therapy that offers a targeted approach to the cell cycle perturbations of malignancy.

In this study AT7519, a novel CDK inhibitor, showed efficacy as a single agent and in combination with gemcitabine in vitro having anti-proliferative effects against a range of pancreatic cancer cell lines. In vivo AT7519 demonstrated efficacy and tolerability both as a single agent and in combination with gemcitabine. AT7519 is a potential agent that warrants further investigation in pancreatic cancer and may be beneficial in reducing the issues currently faced with chemoresistance standard therapies.

HSP90 is involved in the regulation of many oncogenic proteins and its inhibition and the subsequent effects of multiple molecules and pathways is an attractive prospect in cancer therapy. This is particularly relevant to pancreatic cancer, as more is understood about the cancer biology the complex and multiple pathways

that are deranged, these need to be targeted in order to improve the poor outcomes that current chemotherapeutics provide.

In this study AT13387, a novel HSP90 inhibitor, showed activity in vitro against pancreatic cancer cell lines including one with acquired gemcitabine resistance and down stream effects consistent with HSP90 inhibition were observed. In vivo AT13387 slowed tumour growth as a single agent but performed poorly in combination with gemcitabine. The data presented does not support the addition of At13387 to current gemcitabine therapy but does offer some possibilities as an alternative therapy for patients who are non-responders to current therapies and may be of benefit in combination with other targeted treatments.

In light of the ongoing poor prognosis and response to current chemotherapeutics pancreatic cancer continues to pose a significant health problem and is in urgent need of new and novel therapies which offer better outcomes for the patients affected. Combining treatments is desirable as previously discussed with current understanding of the multiple pathways related to pancreatic cancer. Combining chemotherapeutic agents has been this far disappointing with no significant improvements in overall survival but increased toxicity. More research is needed on the reasons for resistance to existing therapies and further clinical trials on combinations of gemcitabine with agents that would target cells resistant to gemcitabine. Combining standard chemotherapeutic agents with novel therapies

that have a different mechanism of action offer an avenue to overcome these limitations.

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APPENDIX: PUBLISHED WORK

1. **Thomas A**, Shaw E, Ghaneh P, Greenhalf W, Costello E, Dejani K, Lyons J, Sibson R, Neoptolemos J. Assessment of the CDK inhibitor AT7519 in a xenograft model of pancreatic cancer. NCRI 2011; 7: A61. (Abstract publication).

2. Shaw E, **Thomas A**, Ghaneh P, Greenhalf W, Davies M, Costello-Goldring E, Gibbs F, Lyons J, Neoptolemos J, Sibson R. Inhibition of tumour growth in a pancreatic cancer xenograft model by AT13387, an HSP90 inhibitor. NCRI 2011; 7: A60. (abstract publication).

3. **Thomas A**, Shaw E, Ghaneh P, Greenhalf W, Costello E, Dajani K, Squires M, Sibson R, Neoptolemos.J. AT7519, a Novel CDK Inhibitor, Is Effective Against Gemcitabine Resistant Pancreatic Cancer Cell Lines. Pancreatology. 2011;11:203. (abstract publication).

4. Shaw E, **Thomas A**, Ghaneh P, Greenhalf W, Davies M, Costello E, Gibbs F, Squires M, Sibson R, Neoptolemos J. AT13387, a Novel HSP90 Inhibitor Induces Cytostatic Effects in Pancreatic Cancer Cell Lines. Pancreatology 2011;11:204. (abstract publication).

5. **Thomas A,** Greenhalf W, Shaw E, Jones O, Costello E, Dejani K, Davies M, Lyons J, Sibson R, Neoptolemos JP, Ghaneh P. AT7519, A novel CDK inhibitor is effective against pancreatic cancer cell lines and in a pancreatic cancer Xenograft model (pending submission)

ORIGINAL PUBLICATION (PENDING SUBMISSION)

AT7519, A novel CDK inhibitor is effective against pancreatic cancer cell lines and in a pancreatic cancer Xenograft model

Thomas A, Greenhalf W, Shaw E, Jones O, Costello E, Dejani K, Davies M, Lyons J, Sibson R, Neoptolemos JP, Ghaneh P

Abstract

Background

Pancreatic cancer continues to carry a poor prognosis. Chemotherapy is used following surgery and in advanced disease; though current agents e.g. gemcitabine+capecitabine or FOLFIRINOX, provide modest survival benefit. There is an urgent need for more effective therapies. We evaluated a novel CDK inhibitor, AT7519, in pancreatic cancer.

Methods

Cell proliferation assays for IC50 values and isobolar analysis were performed using EZ4U assay. Cell cycle analysis was performed with flow cytometry. Standard western blot analysis with ECL detection was completed to assess downstream markers. In vivo studies were conducted using a murine xenograft model. Tumour volume was assessed with external callipers. Experiments were performed using AT7519 as a single agent and in combination with gemcitabine.

Results

AT7519 was tolerated as a single agent and in combination with gemcitabine. Mice treated with AT7519 showed decreased tumour growth (p0.0446) compared with control and studies in combination with gemcitabine are near completion. AT7519 inhibited proliferation in pancreatic cancer cell lines including gemcitabine resistant lines. AT7519 and gemcitabine in combination displayed an additive effect and S phase blockade. Downstream effects were observed with inhibition of phosphorylation of Rb, NPM and pp1-α.

Conclusion

AT7519 is a promising agent for combination therapy in pancreatic cancer.

Introduction

Pancreatic cancer continues to a pose significant health problem being the 6th most common cause of cancer death in the UK equating to around 7 700 deaths per year cancer. Despite current research and advances in understanding pancreatic cancer biology, imaging detection methods and improved surgical outcomes, the prognosis remains poor with a five-year survival of around 10% in resected disease. Despite advances in many other cancers leading to improved survival the incidence and mortality rates for pancreatic cancer have not seen any significant improvement over the last 30 years and are predicted to remain static in Europe in 2012(Malvezzi, Bertuccio et al. 2012).

The basis of current therapies for PDAC involves targeting of DNA replication by inhibition of thymidylate synthase and incorporation of fluorouridine derivatives into nascent DNA strands. The agents that are used are 5-FU, capcetabine and gemcitabine and also have an effect on transcription and DNA methylation. These therapies are used in both the adjuvant and advanced disease setting based on previous trial evidence (Burris, Moore et al. 1997; Neoptolemos, Stocken et al. 2004; Oettle, Post et al. 2007; Sultana, Smith et al. 2007; Neoptolemos, Stocken et al. 2010; Conroy, Desseigne et al. 2011). Despite the demonstrated survival benefits recurrence rates in resected disease remain high and in all disease settings long-term survival remains poor due in part to chemoresistance. Cancer cells become resistant to therapy by a number of hypothesised means: by exclusion of drugs from the cancer cells, by changes in enzymes metabolising drug or by becoming resistant to apoptosis and cellular stress. Further increases in survival should be possible by targeting these areas that lead to resistance or targeting multiple pathways in combination with standard therapy. Jones et al identified 12 pathways commonly mutated in PDAC(Jones, Zhang et al. 2008). Clearly this underpins the complex genetic anomalies that exist in PDAC and supports the concept that targeting single genes or proteins will likely be ineffective. Therapy that targets multiple pathways at different levels in combination with standard therapy may offer the most chance of making significant differences to survival of patients. Genetic mutations that arise in pancreatic cancer often have effect on crucial checkpoints within the cell

cycle and CDK's being pivotal to cell cycle regulation could offer another target for inhibition in pancreatic cancer. CDK inhibition could offer future promise for therapy in PDAC.

The replication of DNA and mitosis are reliant on the activity of cyclin-dependant protein kinase (CDK) enzymes. CDK's form heterodimers with a cyclin subunit and the activity of this molecular complex governs cell cycle regulation.

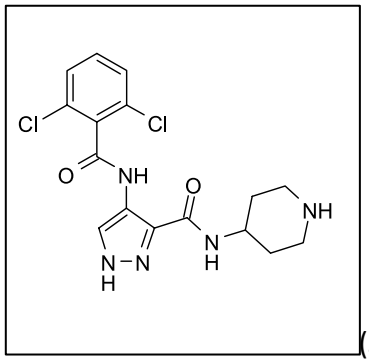
Cancer cells, by definition, are prone to inappropriate cell division and the activity of CDK's and cyclins are frequently deranged in cancer. This may be due to amplification of genes encoding for specific CDK's or cyclins, other protein overexpression mechanisms, gene deletion or gene silencing of a CDK inhibitor such as p16INK4a(Malumbres and Barbacid 2001).

Relationships between cdk's cell cycle regulation and tumour suppressor genes are critical in cancer. Aberrant pRb signalling pathways are common in the majority of human cancers resulting in unchecked entry into S phase. Overexpression of cyclin D1 or deletion of p16INK4A activates the pRb pathway in this manner. P53 is an essential regulator of the G1 checkpoint and arrests growth through induction of p21 and G2 arrest by promoting transcription of GADD45 that inhibits cyclin B-CDK1 activation.

The role of aberrant CDK activity and potential inhibition has been investigated in many human cancers. Novel CDK inhibitors have also been investigated in PDAC. Brasca et al reported on a novel CDK inhibitor PHA-793887 and its effects in a xenograft model of pancreatic cancer using the tumour line Bx-PC3. The agent was well tolerated and resulted in significant tumour growth inhibition (Brasca, Albanese et al. 2010).

AT7519 is a general CDK inhibitor developed by Astexs pharmaceuticals (Cambridge, UK). This compound was developed using fragment-based X-ray crystallographic screening. Fragments are low-molecular weight compounds with low binding affinities and screening using X-ray crystallography yields small libraries of compounds that have much greater probability of yielding complimentary

fragments and targets(Hartshorn, Murray et al. 2005). Astexs developed a panel of molecules and investigated pharmacokinetic properties leading to the identification of AT7519(Wyatt, Woodhead et al. 2008).



(Squires, Feltell et al. 2009)

AT7519 is a potent inhibitor of CDK1, 2, 4, 6 and 9 and also inhibits CDK 3 and 7 to a lesser degree(Squires, Feltell et al. 2009). Initial in vitro experiments across a range of cancer cell lines confirmed the antiproliferative activity of AT7519 and TUNEL staining assays and colony formation confirmed induction of apoptosis in colonic and ovarian cancer cell lines, interestingly an exposure equivalent or greater than one cell cycle was required for induction of apoptosis suggesting that AT7519 needed to be present during certain points in the cell cycle. In this initial investigation a xenograft model using HCT116 (a colonic cancer cell line) was carried out that showed delayed tumour growth and tumour regression in an advanced cancer model compared to controls ($p<0.05$). CDK2 substrates were also tested from this xenograft model, namely the phosphorylation of NPM and Rb and were shown to decrease after certain time points post dose.

The key to the future of chemotherapy in pancreatic cancer will be the identification of novel and effective agents, better biomarker technology underpinned by translational research, which will inform the design of future trials. Ultimately this will ensure that patients will be able to receive targeted therapy to achieve the most benefit.

In this study were investigated the efficacy of AT7519 in pancreatic cancer cell lines and in a pancreatic cancer xenograft model as a potential novel therapy to be

employed in combination with standard clinical agents currently used in the treatment of pancreatic cancer.

Materials and methods

Cell lines and reagents

Pancreatic cancer cell lines SUIT-2, Miapaca-2, CFpac, Fampac and paca2 were derived from stores in the department of Clinical and Molecular Cancer Medicine, University of Liverpool. Panc-1 was supplied by LGC standards from the ATCC. SUIT-2 (GR) (a gemcitabine resistant pancreatic cancer cell line derived from the parent line SUIT-2 was developed in the department of molecular and clinical cancer medicine by K Dejani. Cells were grown in RPMI supplemented with 10% foetal bovine serum and maintained at 37^{o}C in an atmosphere of 5% CO2. Pancreatic cell lines were cultured as previously described.

AT7519 is N-(4-piperidinyl)-4-(2,6-dichlorobenzoylamino) 1H-pyrazole-3-carboxamide. AT7519 was obtained from Astex Pharmaceuticals Ltd (Cambridge, UK). It was dissolved first in dimethyl sulfoxide (DMSO; Sigma Chemical, USA) at a concentration of 10 mM, and then in culture medium immediately before use. Gemcitabine was obtained from stores in the department of clinical and cancer medicine, University of Liverpool. It was dissolved in PBS (sigma Chemical, USA) at a concentration of 0.1M and in culture medium immediately before use.

Cell viability and proliferation assays

AT7519's effects on viability of PDAC cell lines and isobolar analysis of AT7519 in combination with gemcitabine was assessed using the cell proliferation assay EZ4U (biomedical) measured at 4, 24, 48, 72 and 96 hrs after drug treatment and performed in triplicate. Cells were seeded into 96-well plates at 1.5 x 104 per well and cultured for 24 hrs before the addition of the agent or control in 0.1% DMSO at varying concentrations.

Isobolar analysis

An assessment of synergy (or antagonism) was carried out using isobolographic analysis (berenbaum 1989, tallarida 1989 1992). Cells were plated and cultured as per IC50 cell proliferation assays above and cells were treated with variable concentrations of AT7519 and gemcitabine around the established IC50 values for single agents or control. The isobolar analysis was also performed with sequential addition of agents with gemcitabine initially then addition of AT7519 after 24 hours. The results were standardised against controls and an isobolar curve generated to display the relationship between the agents in combination by plotting a straight line connecting the two agents IC50 points representing an additive effect. The data plotted indicted the effect of the drugs in combination, if below the additive line synergy was demonstrated, above the line antagonism and on the additive line the drug effects were purely additive i.e. no interaction.

Cell cycle analysis

Cell cycle analysis was performed using propidium iodide (PI) staining and flow cytometry. Miapaca-2 cells were seeded onto 6 well plates at 2.5 x 105 cells per well, grown overnight and then treated with AT7519, gemcitabine (+/-) or control (0.1% DMSO or PBS) for various time points at doses detailed in figure legends. Both adherent and floating cells were collected and washed with twice with PBS prior to fixation in 70% ethanol overnight before staining with PI (50ug/ml) for 15minutes at room temperature. Propidium iodide incorporation using assessed using a CyAn™ ADP Analyzer (Beckman Coulter). Duplicates were formed each time and experiments were repeated in duplicate on different days.

Detection of apoptosis

To determine the timing and mechanism of cell death caspase 3/7 activity was assessed using the Caspase-Glo 3/7 luminescent assay (promega). Miapaca-2 cells were seeded at 5000 cells/ well in 96 well plates, left overnight to adhered and then treated with 10uM AT7519 or 0.1% DMSO control for 6, 16, 24, 40, 48 or 72 hours. Caspase-Glo reagent was added, as detailed in the manufacturers protocol using 100ul volumes and incubated for 1 hour. Luminescence was then measured in a microplate reader and normalised to untreated control. A single experiment was performed with 6 replicates for each time point.

Western blotting

Pancreatic cancer cells were cultured with AT7519 a variable concentrations, harvested, washed and lysed using lysis buffer as previously described by Astexs pharmaceuticals (ref). The protein concentration of lysate was measured, mixed with gel electrophoresis loading buffer, boiled for 5 min, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to nitrocellulose membrane. The membranes were blocked in PBS plus 5% non fat milk powder and 0.1% Tween 20 for 1 h before incubating with the antibodies overnight at 37oC. Immunoblotting was done with cdc2, Rb, pRb from Cell signalling Technology. Decetction was performed using ECL reagents. Blots were stripped and reprobed with b-actin (Santa Cruz Biotechnology) antibodies to ensure equal protein loading.

Xenograft mouse model

To evaluate the in vivo tolerability and efficacy of AT7519, gemcitabine and combination therapy BALB/c (CAnN.Cg-Foxn1nu/Cr) nude mice were used (Harlan UK, housed in pathogen free conditions, 6-8 weeks old). In tolerability studies non-

tumour bearing mice were used and treated for two weeks with AT7519 7.5mg/kg BD 5 days per week, gemcitabine 50 or 75mg/kg 2qw or AT7519 7.5mg/kg BD 5 days per week + gemcitabine 50mg/kg 2qw. Body weights and general observations were performed daily, experimental endpoints were a loss of >20% of day 1 body weight in two or more animals (sustained over a 72 hr period) or death of more than one animal. Animals were not dosed if their was a >15% body weight loss. Necropsy for serum and organs was performed. For Efficacy studies mice were inoculated with Miapaca-2 pancreatic cancer cells. Miapaca-2 were supplied by LGC standards from the ATCC that had been cultured and frozen in stocks adequate for all in vivo studies then defrosted and cultured in T75 flasks until 90% confluence and no signs of infection. Cells were then harvested and diluted in PBS to a concentration of 1 x 107/ml and added to an equal volume of matrigel and kept on ice until subcutaneous inoculation of each mouse with 200ul into the shoulder area bilaterally. When tumours were palpable/measurable, mice were treated i.p. with saline 0.9% vehicle, AT7519 7.5mg/kg BD 5 x per week or gemcitabine50mg/kg 2qw ; or a combination of AT7519 and gemcitabine at the same doses. Tumour size was measured every alternate days in three dimensions using callipers, and tumour volume was calculated. The experimental endpoints were tumours reaching a diameter of $17mm^3$ or became ulcerated, loss of >20% of day 1 body weight in two or more animals (sustained over a 72 hr period) or death of more than one animal. Animals were not dosed if their was a >15% body weight loss. Survival and tumour growth were evaluated from the first day of treatment until death. Necropsy was performed for tumour, serum and organs. All animal studies were approved by the Home office animals in scientific procedures act.

Analysis

All in vitro experiments were performed in triplicate and repeated at least 3 times; a representative experiment was selected for Figures. Statistical significance of differences for both in vitro and in vivo experiments were determined using

Student's t-test, with minimal level of significance p=0.05. All statistical analyses were determined using SPSS.

Results

AT7519 inhibited the proliferation of pancreatic cancer cell lines with IC50 values ranging from 0.18– 2uM (figure 1)

AT7519 ability to inhibit cell proliferation was assessed in 7 pancreatic cancer cell lines. Cell lines sensitive and resistant to conventional therapy were assessed. Cells were cultured with increasing concentrations of AT7519 (50nm - 5000nM) for 24, 48 and 72 hours. The IC50 values ranged from 180nM to 2uM (72 hour reading IC50 shown). The gemcitabine resistant cell line Suit-2 GR had was found to have an IC50 of 625nM to gemcitabine compared to 10nM in the parent line Suit-2. The AT7519 IC50 was lower in the gemcitabine resistant cell line (700nM) than the parent line (2uM) suggesting the resistant mechanism for gemcitabine did not overlap with resistance to AT7519.

Cell line	IC50 AT7519	IC50 Gemcitabine
SUIT-2	2uM	10nM
SUIT-2 (GR)	700nM	625nM
Miapaca-2	390nM	25nM
Panc-1	275nM	150nM
CFpac	180nM	180nM
FamPac	210nM	5.5nM
Paca 2	250nM	5nM

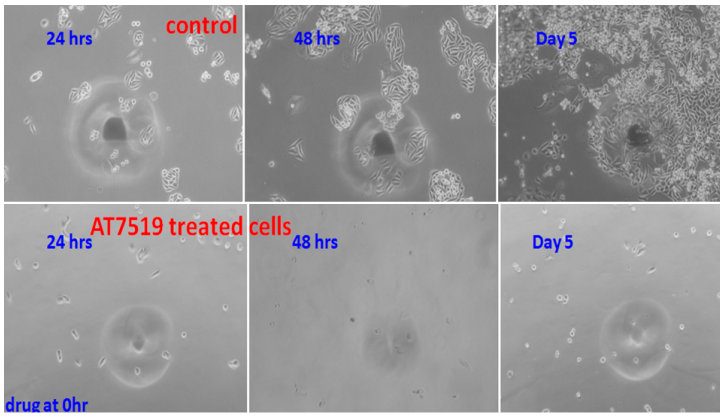
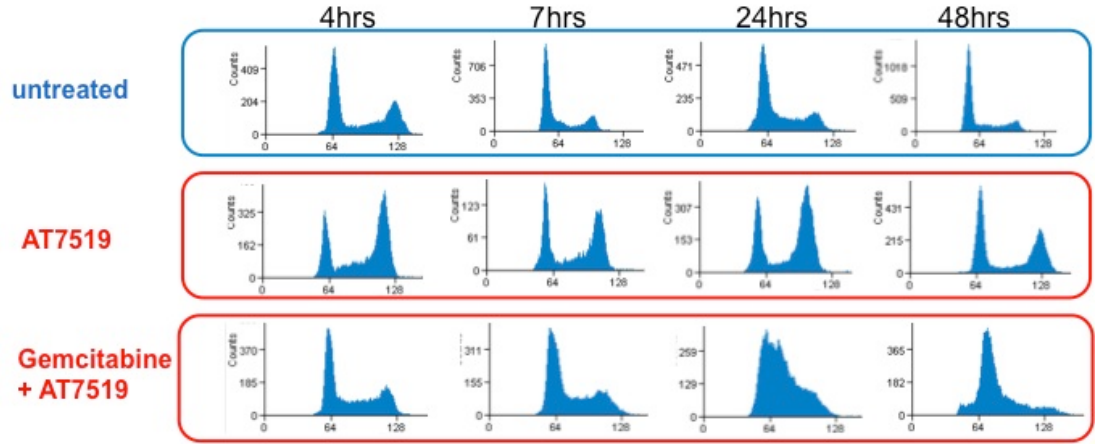


Figure 1. Anti-proliferative activity of AT17519 in vitro
[a] IC50 values across a range of pancreatic cancer cell lines.
[b] Photography of cell proliferation in untreated cells versus AT7519 treated cells (nm) at various time points.

Cell cycle analysis showed a marked reduction of cells in the G0/G1 and S phases with an increase in cells in G2/M phase following treatment with AT7519 (figure 2)

Cell cycle analysis was performed on suit-2 cells cultured with media alone or AT7519 (10uM) for 4, 7, 24 and 48 hours. Following 24 hours treatment with AT7519 there was clear arrest in G2/M phase with a reduction in the proportion of cells in G0/G1 and S phase compared to untreated cells (p=<0.001). When AT7519 was combined with gemcitabine there was an accumulation of cells in S phase in keeping with the effects of gemcitabine alone but AT7519 appeared to enhance this effect.



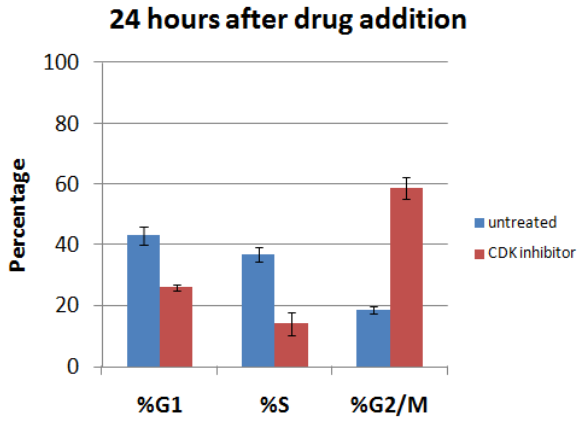


Figure 2.Effects of AT7519 on the cell cycle
[a]Cell cycle distribution of SUIT-2 pancreatic cancer cell line untreated (media only), treated with AT7519 (nm), treated with AT7519 (nm) and gemcitabine (nM) at 4, 7, 24 and 48 hours.
[b] percentage of cells in different phases of the cell cycle treated with AT7519 versus untreated control SUIT-2 cells at 24 hour time point.

Apoptosis was demonstrated after treatment with AT7519 in vitro by caspase-3 activity (figure 3)

Evidence of induction of apoptosis by AT7519 in pancreatic cancer cell lines tested was limited. Caspase-3 activity was investigated and at 24 hours induction of caspase-3 was significantly increased in treated cells compared to untreated control.

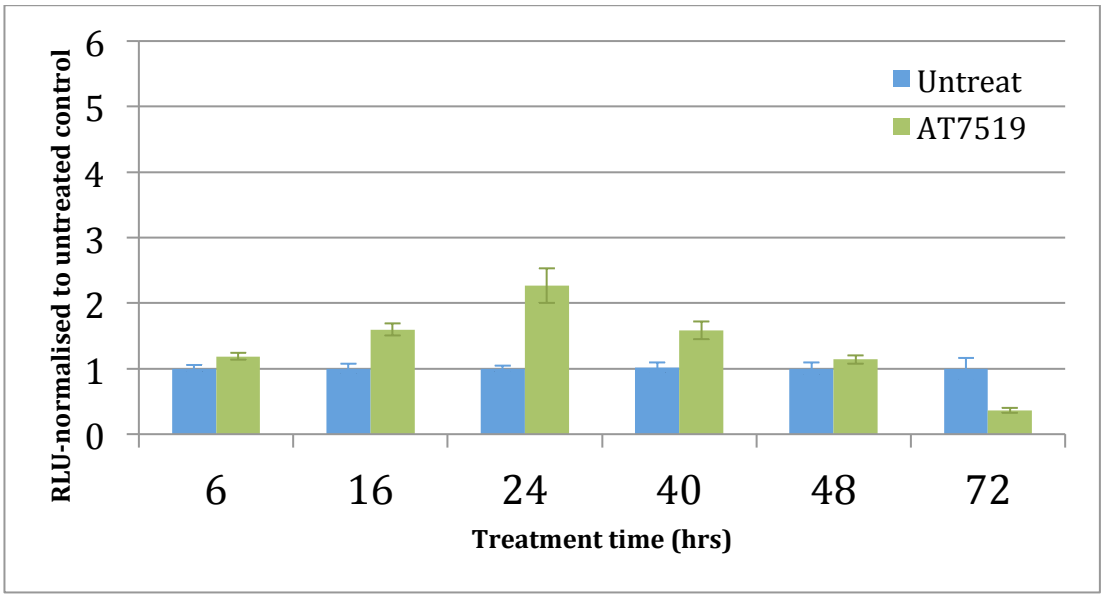


Figure 3. caspase-3 activity of untreated and AT7519 treated Miapaca-2 pancreatic cancer cells at 6, 16, 24, 40, 48 and 72 hours.

Isobolar analysis of AT7519 combined with gemcitabine suggested an additive effect (figure 4)

Initial experiments performed demonstrated an antagonistic effect when AT7519 and gemcitabine was given simultaneously (data not shown) consistent with a need for cells to be dividing in order for gemcitabine to have an effect. In light of this experiments were performed giving gemcitabine alone for the first 24 hrs of incubation followed by the addition of AT7519. These experiments demonstrated at least an additive effect when AT7519 and gemcitabine where given in combination with sequential dosing.

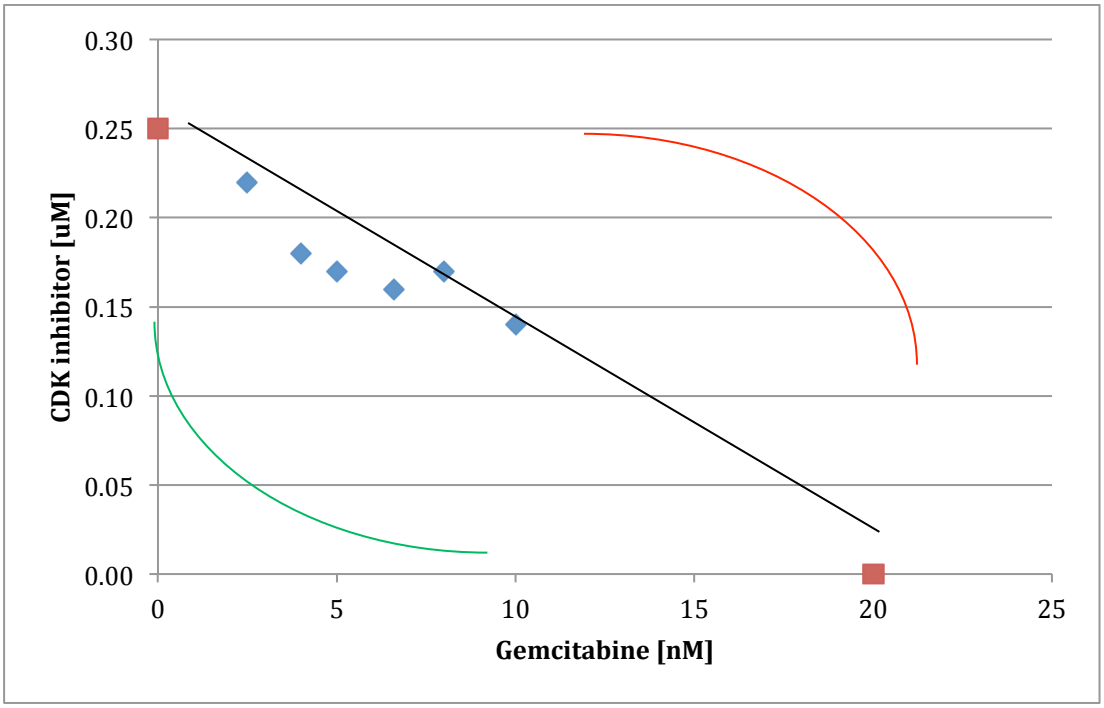
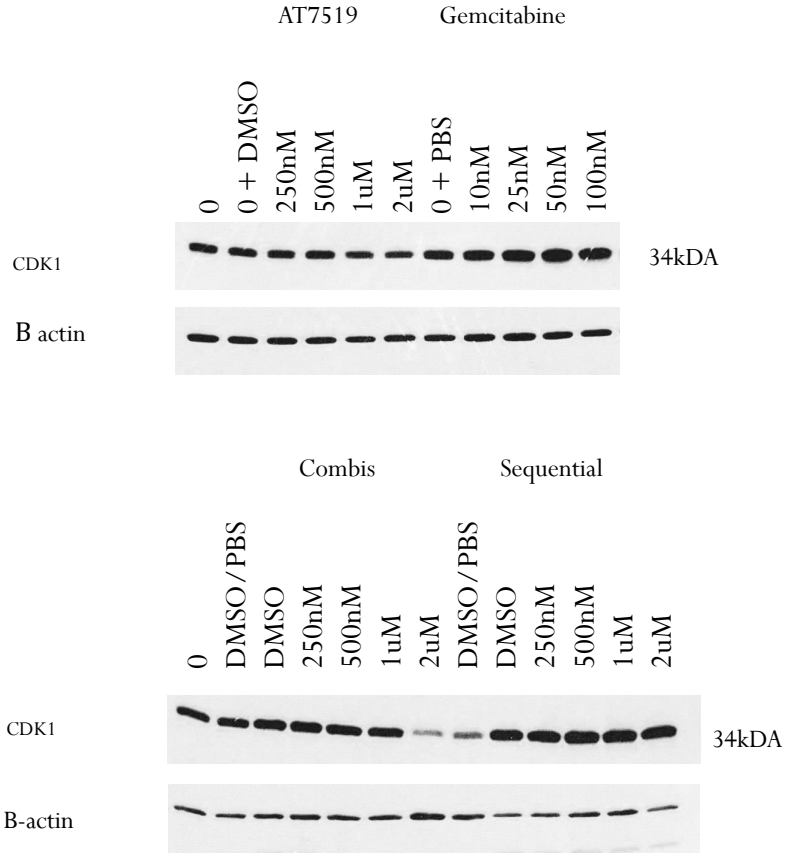


Figure 4. Isobolar graph of cells treated with gemcitabine for 24 h followed by combination of gemcitabine + AT7519 for 48 h.
The line represents an additive effect. The area under the curve would be lower if there was synergy and greater if there was antagonism

Western blot analysis demonstrated the effect on downstream targets of AT7519 (figure 5)

Entry of cells into mitosis is regulated by CDK1 kinase activation. Cell cycle analysis supports an AT7519 G2/M block (as expected if CDK1 activity is inhibited). Western blot analysis confirmed decreased CDK1 levels further supporting the G2/M block effect of AT7519 not observed with gemcitabine as a *single* agent. When gemcitabine and AT7519 were used in combination this decrease in CDK1 was not observed supporting the notion that cells need to be dividing in order for G2/M block to occur. This was confirmed by sequential addition of gemcitabine alone for 24 hours followed by AT7519, this lead to appropriate variation of CDK1. Phosphorylation of Rb is catalysed by cyclin-dependant kinases and inhibited by AT7519. Rb phosphorylation is almost eliminated despite minimal changes in Rb levels.



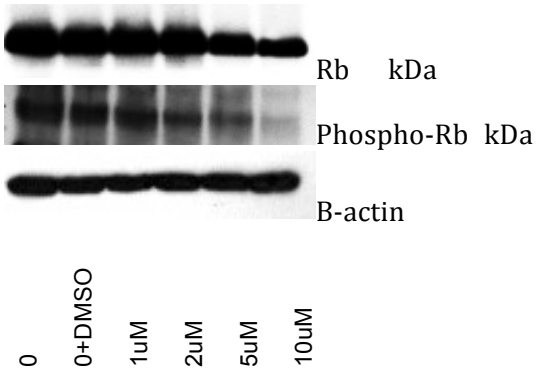


Figure 5. [a]Western blot showing the downstream effects on cdc2 of AT7519 and gemcitabine as single agents
[b]Combination: 25nM Gemcitabine and desired concentration of AT7519 added together
Sequential: Cells treated with 25nM of Gemcitabine for 24h followed by the addition of the desired concentration of AT7519
[c] Western blot showing effect of AT7519 (1uM-10uM) on Rb and phosph-Rb antibodies

AT7519 was well tolerated as a single agent and in combination with gemcitabine in nude mice (figure 6)

To test the tolerability (and establish gemcitabine dosing) non-tumour bearing nude mice were treated with single agents and combination therapy for 2 weeks with 3 mice per group. AT7519 was tolerated well as a single agent at 7.5mg/kg BD 5/7 days and in combination studies. One animal died due to an injury unrelated to AT7519 treatment. Gemcitabine was tolerated at 50mg/kg 2qw both as a single agent and in combination studies (data not shown).

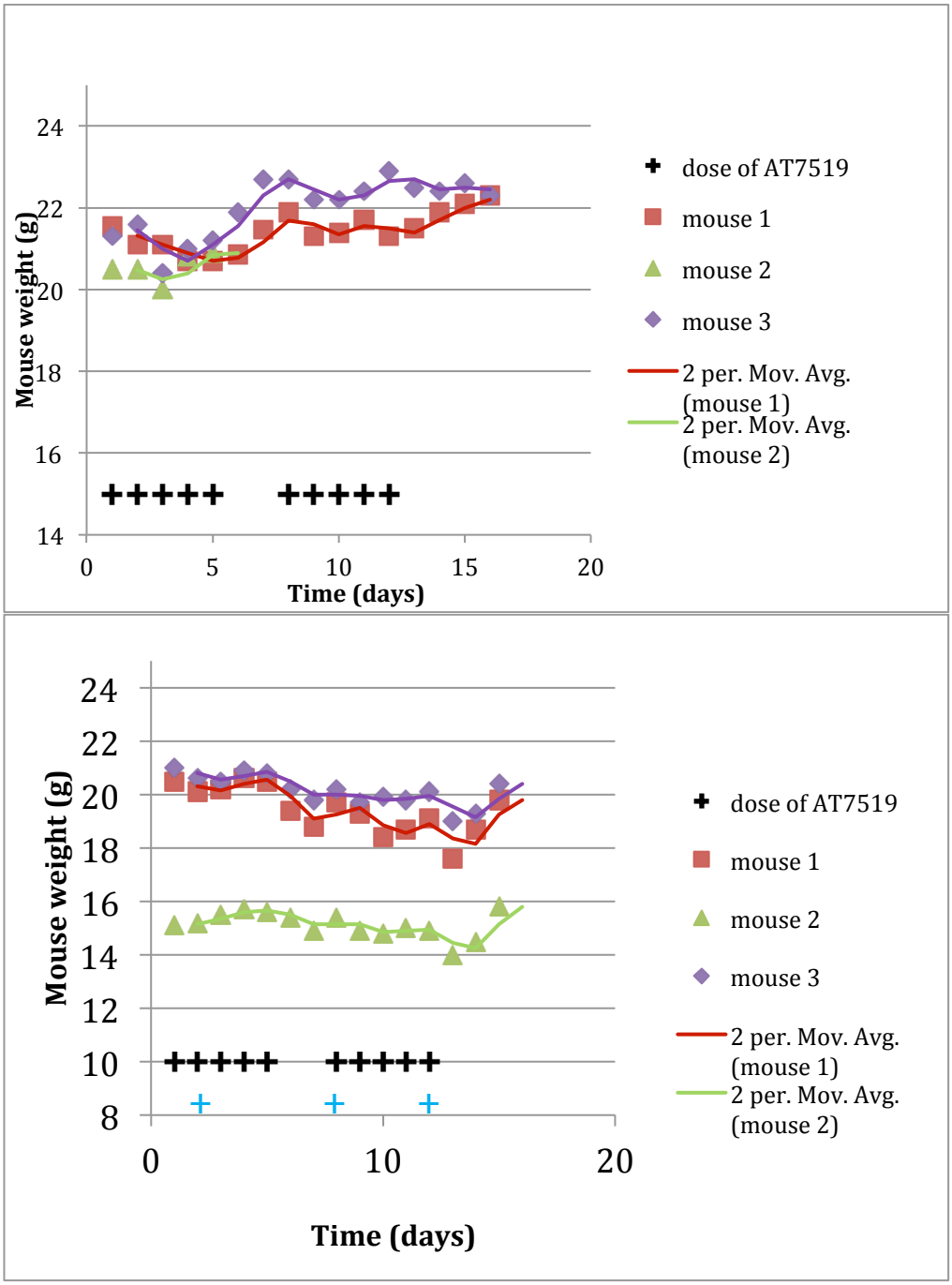
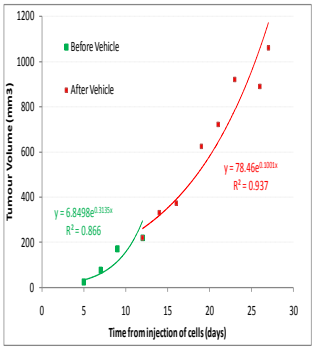


Figure 6. [a] Tolerability of AT7519 as a single agent, graphical representation of weight [b]Individual mouse weights (g) for 3 mice dosed with 50mg/kg of gemcitabine twice per week and 7.5mg/kg of AT7519 twice daily for 5 days. Total treatment of 2 weeks.

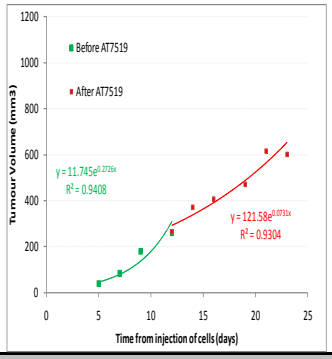
In a *xenograft model of pancreatic cancer, AT7519 as a single agent and in combination with gemcitabine showed a significant reduction in tumour growth compared to vehicle control (figure 7)*

SUIT-2 pancreatic cancer cell line were successfully cultured and developed subcutaneous solid tumours in nude mice with matrigel. Tumours grew in an exponential fashion. From tolerability studies animals were dosed appropriately with AT7519, gemcitabine, vehicle control (saline) or a combination of AT7519 and gemcitabine. During the single agent efficacy study AT7519 significantly reduced the exponential tumour growth ($p=0.043$) as did gemcitabine ($p=0.0038$). There was no significant body weight loss in the single agent dosing of AT7519. In the combination efficacy study of AT7519 and gemcitabine a further beneficial effect of reduction in tumour growth was observed that was superior to vehicle control ($p=0.0041$) either AT7519 ($p=0.0173$) or gemcitabine alone ($p=0.0611$).

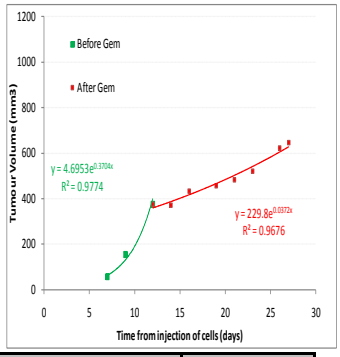
(A) Saline Control



(B) AT7519



(C) Gemcitabine

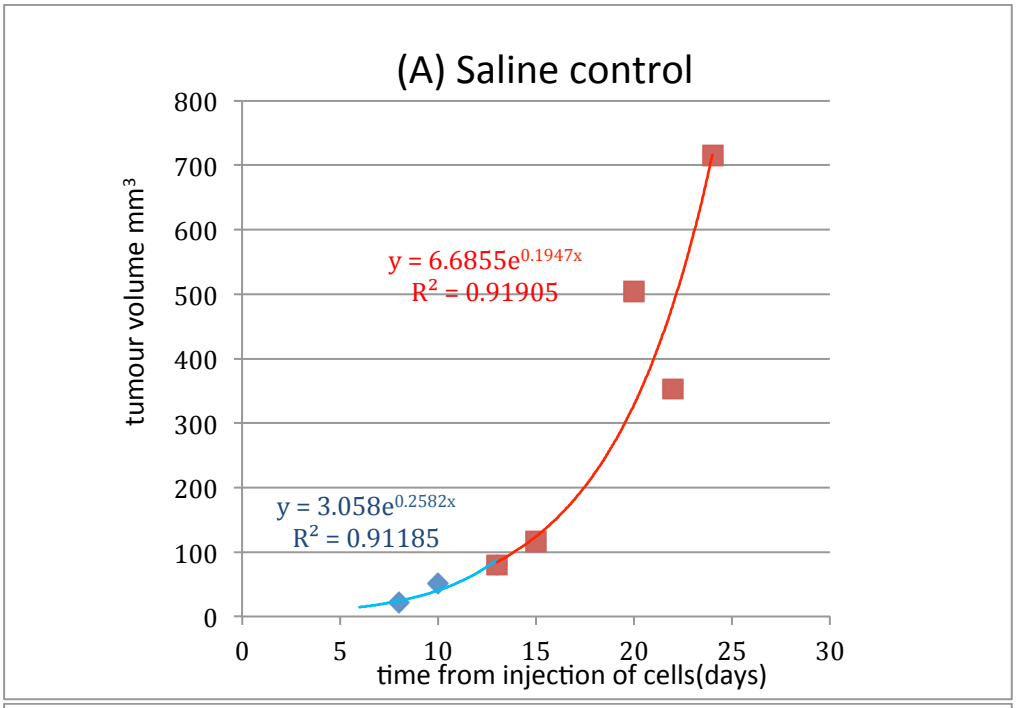


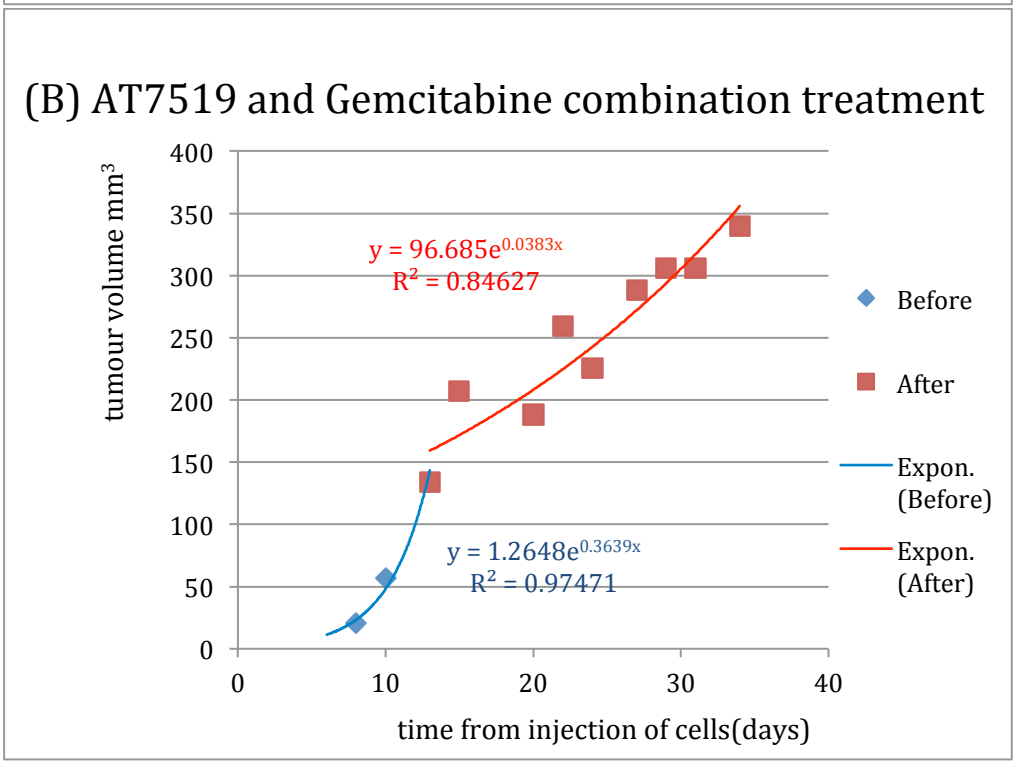
Treatment	Mean coefficient difference	N	P value
Vehicle control	0.162	6	0.502
AT7519	0.29	6	0.045
Gemcitabine	0.445	6	0.038

Figures 7:

[a]Tumour volumes in mice treated with Saline vehicle control(A), AT7519 (B) and Gemcitabine (C) with log regression analysis co-efficient plotted.

[b] table of Log regression analysis coefficients mean difference after treatment for each group with p values of significance.


(A) Saline control
tumour volume mm3
800
700
600
500
400
300
200
100
0
y = 6.6855e0.1947x
R² = 0.91905
y = 3.058e0.2582x
R² = 0.91185
0
5
10
15
20
25
30
time from injection of cells(days)


(B) AT7519 and Gemcitabine combination treatment
tumour volume mm3
400
350
300
250
200
150
100
50
0
y = 96.685e0.0383x
R² = 0.84627
y = 1.2648e0.3639x
R² = 0.97471
0
10
20
30
40
time from injection of cells(days)
Before
After
Expon. (Before)
Expon. (After)

Treatment	*Mean coefficient difference*	*N*	*P value compared with control*	*P value compared with gem*	*P value compared with AT7519*
Vehicle	*0.05*	*6*	*-*	*0.9991*	*0.9906*
Gemcitabine	*0.162*	*6*	*0.0198*	*-*	*0.1477*
AT7519	*0.114*	*6*	*0.029*	*0.9374*	*-*
AT7519 + Gemcitabine	*0.249*	*6*	*0.0041*	*0.0611*	*0.0173*

Figures 8: [a]Tumour volumes in mice treated with Saline vehicle control(A) and Gemcitabine + AT7519 (B).
[b] Table of mean coefficient difference for each group before and after treatment with p-values for paired T-test comparing co-efficient change.

Discussion

Pivotal to the regulation of the cell cycle are cyclins and cyclin dependant kinases. Cancer cells by definition are subject to inappropriate cell division and aberrant CDK activity has been documented as one of the hallmarks of cancer (Hanahan and Weinberg 2011). The investigation of CDK inhibition and small molecule novel agents such as AT7519 is appropriate and may improve survival in PDAC(Lapenna and Giordano 2009). The relative chemoresistance of pancreatic adenocarcinoma to standard therapy remains a current problem and at least partly explains the relatively poor survival associated with PDAC both in the resectable and advanced setting. With greater understanding of cancer biology and the need for new approaches novel therapies remain an important avenue to attempt to overcome these issues. AT7519 a novel CDK inhibitor investigated here shows efficacy in vitro against a range of pancreatic cancer cell lines and activity against a gemcitabine resistant cell line. In cell cycle analysis AT7519 showed activity in line with expected CDK inhibition as for mentioned an important pathway in cancer biology and its cell cycle effects in combination with gemcitabine offers important information regarding the dosing schedule to devise maximal efficacy of this agent in combination with gemcitabine. The cytotoxicity of AT7519 has been demonstrated

by in vitro assays and in cell cycle studies and apoptosis demonstrated by caspase 3 activity. When downstream markers were investigated the effects of AT7519 on key pathways in the cell cycle was demonstrated and again the importance of dose scheduling highlighted. The importance of scheduling AT7519 with gemcitabine to allow both agents to take maximal effect was further demonstrated by isobar analysis supporting the principle that gemcitabine needs cells to be diving in order to have an effect. In a murine model AT7519 was well tolerated and showed efficacy both as a single agent and in combination with gemcitabine significantly reducing tumour burden.

In targeting multiple pathways general CDK inhibitors such as AT7519 provide a promising therapeutic option excerpting both direct cell cycle and transcription effects(Malumbres 2012), evidence of actions on some of these known aberrant pathways have already been investigated. Fry et al demonstrated the activity of PD 000332991 on tumour xenografts, this CDK inhibitor specifically targets CDK4 and 6 and therefore the downstream phosphorylation of Rb(Fry, Harvey et al. 2004). More recently a novel CDK inhibitor Dinaciclib was demonstrated to inhibit pancreatic cancer progression in a murine model and its CDK5 inhibition hypothesised to affect Ras-mediated Ral activation(Feldmann, Mishra et al. 2011). Regarding the use of CDK inhibitors in pancreatic cancer the important question remains that which CDKs or range of CDKs should be target and would provide the optimum therapeutic option for this disease. As the knowledge on the genetic basis of pancreatic adenocarcinoma becomes clearer this question will be more readily answered, certainly the effects demonstrated by AT7519 offer promise and suggest that at least partly this selective CDK inhibitor offers a useful therapeutic option as an adjunct to standard therapy. The information to date on CDK inhibitors support the notion that using these agents in combination rather than monotherapy offers the most desirable therapeutic avenue.

Combining therapies is desirable as previously discussed with current understanding of the multiple pathways related to pancreatic cancer(Jones, Zhang

et al. 2008; Greenhalf and Thomas 2011). More research is needed on the reasons for resistance to existing therapies and further clinical trials on combinations of gemcitabine with agents that would target cells resistant to gemcitabine.

Conclusion

The key to the future of adjuvant therapy in pancreatic cancer will be the identification of novel and effective agents, better biomarker technology underpinned by translational research that will inform the design of future trials. AT7519 inhibited growth in pancreatic cancer cell lines including one that has acquired gemcitabine resistance and was effective in combination with gemcitabine both in vitro and in vivo. AT7519 is a promising agent for further investigation to overcome the problem of acquired gemcitabine resistance in pancreatic cancer.

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